COLONIZACIÓN MICROBIANA Y SUCESIÓN PRIMARIA EN SUELOS DESCUBIERTOS TRAS EL RETROCESO DE GLACIARES EN TIERRA DEL FUEGO, CHILE

Tesis Doctoral

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A mis padres, por todo

A Cristina

"Nada es permanente, a excepción del cambio"

Heráclito

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INTRODUCCIÓN GENERAL

"La vida es como montar en bicicleta:

para mantener el equilibrio solo hay que seguir pedaleando"

Albert Einstein

A. INTRODUCCIÓN GENERAL

A.1 Sucesión ecológica

Los ecosistemas no son elementos estáticos, sino que, de manera natural, evolucionan a lo largo del tiempo, debido tanto a procesos relacionados con su dinámica interna así como a factores externos. El proceso de cambio de la composición y estructura de las comunidades biológicas es lo que se conoce como sucesión ecológica (Pielou 1966; Golley 1977; Luken 1990; Walker y del Moral 2003). Estos procesos pueden iniciarse con la colonización de hábitats recientemente formados o con la recolonización de otros que se han visto afectados por una perturbación muy intensa (Drury y Nisbet 1973; Connell y Slatyer 1977; Pickett et al. 1987; Young et al. 2001; Atlas y Bartha 2002; Walker y del Moral 2003).

El estudio de la sucesión en los ecosistemas terrestres es una disciplina dentro de la ecología que cuenta con más de un siglo de historia. Los primeros trabajos se deben a Warming (1895, 1909), quien realizó una síntesis inicial del proceso de sucesión basándose en las características diferenciales de poblaciones vegetales de distintas superficies recién expuestas, tales como dunas, zonas volcánicas o terrenos en los que se había producido un corrimiento de tierras. Posteriormente, Cooper (1916) interpretó las diferencias entre las comunidades vegetales existentes en distintas áreas localizadas en Glaciar Bay (Alaska, EEUU) observadas por Muir (1915) como distintas etapas sucesionales. Sin embargo, fue Clements (1916, 1928), estudiando el desarrollo temporal de las comunidades de vegetación a lo largo de las Montañas Rocosas (Canadá, EEUU, México), quien se considera sentó las bases para la gran mayoría de los estudios posteriores de sucesión ecológica (Peet y Christensen 1980; Walker y del Moral 2003). En estos trabajos, Clements enunció su teoría de la sucesión, en la que clásicamente se establecen seis procesos fundamentales: 1) la denudación: conjunto de procesos que crean un nuevo sustrato sobre el que se puede iniciar un proceso de sucesión; 2) la migración: los propágulos de los organismos llegan al sustrato recién creado; 3) la ecesis: establecimiento y crecimiento de estos propágulos; 4) la reacción: cambio que producen los organismos en los componentes bióticos y abióticos de los ambientes en los que se asientan; 5) la competición: los organismos que ocupan un área impiden el

asentamiento de otros; y finalmente, 6) la estabilización o "clímax": resultado último de la sucesión.

Con posterioridad a la teoría clementsiana, Gleason (1926, 1939) propuso una teoría sucesional en la que se da más importancia para el desarrollo de estos procesos a los atributos de los organismos individuales, así como a sus relaciones con el ambiente, en lugar de al funcionamiento conjunto de la comunidad propuesto por Clements. Esta visión es menos dogmática en lo que a los resultados de la sucesión se refiere, ya que propone que las etapas finales de la sucesión serán aquellas en las que se registra un recambio lento de especies, acompañado de cierto equilibrio poblacional, sin que tenga necesariamente que existir un punto final climácico determinado, predecible y completamente estable, como se proponía en la teoría clementsiana. Junto con estas dos grandes corrientes, las ideas propuestas también a principios del siglo pasado por Cowles (el primer ecólogo en proponer la sucesión como un proceso secuencial y direccional en su trabajo de 1901) o Tansley (quién propuso el término "ecosistema", así como se mostró contrario a la visión holística de las comunidades ecológicas basada en las ideas de Clements) fueron de gran importancia y sentaron las bases de numerosos estudios posteriores (Shugart 2013; Christensen 2014; Michaud et al. 2015). A mediados y finales del siglo XX aparecieron trabajos de síntesis en el campo de estudio de la sucesión ecológica, como los realizados por Odum (1969) o Connell y Slatyer (1977). En la actualidad, el avance de las técnicas de estudio que permiten analizar todos los componentes de una comunidad, junto con el aumento exponencial de las capacidades computacionales que se han venido produciendo en las décadas más recientes, ha hecho que la corriente de los últimos estudios se base principalmente en las teorías de Gleason (Colinvaux 1993; Young et al. 2001; Hodkinson et al. 2002; Fukami y Morin 2003; Fierer et al. 2010; Shugart 2013). La repercusión que los estudios de sucesión fueron adquiriendo a lo largo de todo el siglo XX ha llevado a considerar el concepto de sucesión como el segundo más importante en Ecología tras la definición de ecosistema, situándolo a un nivel comparable al que tiene el concepto de evolución dentro de la Biología (Margalef 1968; McIntosh 1999; Michaud et al. 2015). Este hecho, junto al gran número de estudios publicados sobre sucesión ecológica, ha generado una amplia variedad de aproximaciones al propio concepto (Pickett et al. 1987, 2009; Nemergut et al. 2013)

A.1.1 Tipos de sucesión ecológica

Clásicamente se han diferenciado dos tipos de sucesión ecológica, la sucesión primaria y la secundaria (Miles y Walton 1993). La sucesión primaria se refiere al patrón temporal de cambio de las comunidades de organismos de un ecosistema que se produce tras la colonización de un sustrato de nueva formación que no haya sido previamente modificado por ningún organismo de manera significativa (Chapin 1994; del Moral y Wood 1993; Begon et al. 1996; Walker 1999; Walker y del Moral 2003; Vitousek 2004; Wardle et al. 2004). Estos procesos se dan, por ejemplo, en las áreas cubiertas por erupciones volcánicas tras el fin de las mismas (del Moral y Wood 1993; Kitayama et al. 1995; Cutler et al. 2014, Kinney et al. 2015) o en los terrenos que quedan descubiertos tras la desaparición de una capa de hielo por retroceso de un glaciar (Chapin et al. 1994; Sigler y Zeyer 2004; Brown y Jumpponen 2014).

El término sucesión secundaria hace referencia al desarrollo que se da en un ecosistema tras una perturbación importante que ha destruido total o parcialmente las comunidades preexistentes, pero no el sustrato sobre el que éstas se asentaban, provocando por tanto un reinicio de la sucesión a partir de un hábitat previamente colonizado (Horn et al. 1974; Jiménez y Armesto 1992; Begon et al. 1996; Walker y del Moral 2003; Garnier et al. 2004). Ejemplos de estos procesos son el desarrollo de las comunidades biológicas del suelo que se produce tras el abandono de terrenos agrícolas (Toky y Ramakrishnan 1983; Scheu 1990; Garnier et al. 2004; Bautista-Cruz y del Castillo 2005; Pueyo y Beguería 2007; Maharning et al. 2009), tras un incendio (Hanes 1971; Purdie y Slatyer 1976; Rull 1999) o tras la tala de un bosque (Covington 1981; Guariguata y Ostertag 2001).

En ambos tipos de sucesión, los cambios que se producen en las comunidades biológicas desde la colonización inicial, y a lo largo del proceso sucesional, comprenden variaciones tanto en su composición taxonómica como en la diversidad de las mismas (Walker y del Moral 2003; Pickett et al. 2005; Hobbs et al. 2007).

A.1.2 Modelos de sucesión ecológica

Un modelo de sucesión es una construcción conceptual creada para explicar procesos complejos mediante la combinación de los diferentes factores implicados (*e.g.* físicos, químicos, biológicos), así como la relación entre estos y las distintas etapas de

desarrollo sucesional de las comunidades (Pickett et al. 1987). Estos modelos tratan tanto de explicar las trayectorias pasadas, como de predecir futuros desarrollos sucesionales (Walker y del Moral 2003; Molles et al. 2006; Johnson y Miyanishi 2010). Existen distintos acercamientos en el diseño de modelos, los cuales difieren en los factores considerados esenciales para explicar los procesos sucesionales (Pickett et al. 1987; Fierer et al. 2010; Nemergut et al. 2013). Así, se ha diferenciado entre modelos alogénicos y autogénicos atendiendo a una clasificación del tipo de factores implicados (Tansley 1929, 1935; Odum et al. 1971; Fisher 1990).

Los modelos alogénicos proponen que los factores abióticos (*e.g.* clima, régimen de precipitaciones, características fisicoquímicas del medio, *etc.*) son los que tienen una mayor influencia en la sucesión. Los factores abióticos modifican el hábitat y, por consiguiente, los organismos que lo ocupan se ven afectados. Por su parte, los modelos autogénicos proponen que son principalmente los factores bióticos (*e.g.* simbiosis, competencia, predación, parasitismo, *etc.*) los principales responsables de los cambios observados en la sucesión (Tansley 1935; Connell y Slatyer 1977; Smith y Huston 1990; Atlas y Bartha 2002; Walker y Del Moral 2003). Pese a que los procesos sujetos a causas autogénicas se han considerado predominantes en numerosos ecosistemas, especialmente en sucesión primaria, la influencia de factores abióticos en los mismos procesos de sucesión no se puede excluir (McFarland et al. 1999; Ellis y Coppins 2006; Granath et al. 2010). Los factores abióticos podrían tener una mayor influencia en las primeras etapas de la sucesión, durante la colonización y asentamiento de los organismos, para posteriormente, cuando el proceso sucesional ha avanzado, ser las interacciones entre los propios organismos, y de éstos con el sustrato, los factores más relevantes (Tansley 1935; Matthews 1992; Amoros y Wade ,1996; Lyautey et al. 2005; Francis 2006).

A.1.3 Mecanismos de sucesión ecológica

El desarrollo de las comunidades biológicas implica interacciones entre ellas y con el biotopo en el que se establecen, afectando con ello a las capacidades de colonización posterior por parte de otros organismos. Según Connell y Slatyer (1977), estas alteraciones pueden producirse por tres mecanismos, a saber, la tolerancia, la inhibición o la facilitación, los cuales se diferencian en la manera en la que influyen en

los procesos sucesionales (Fig. A1). Los tres mecanismos pueden actuar de manera simultánea en un mismo proceso de sucesión, el cual puede mostrar importantes diferencias en su desarrollo según cuál sea el mecanismo que esté actuando mayoritariamente (Fig. A.1, Pickett et al. 1987; Begon et al. 1996; Chapin et al. 1994).

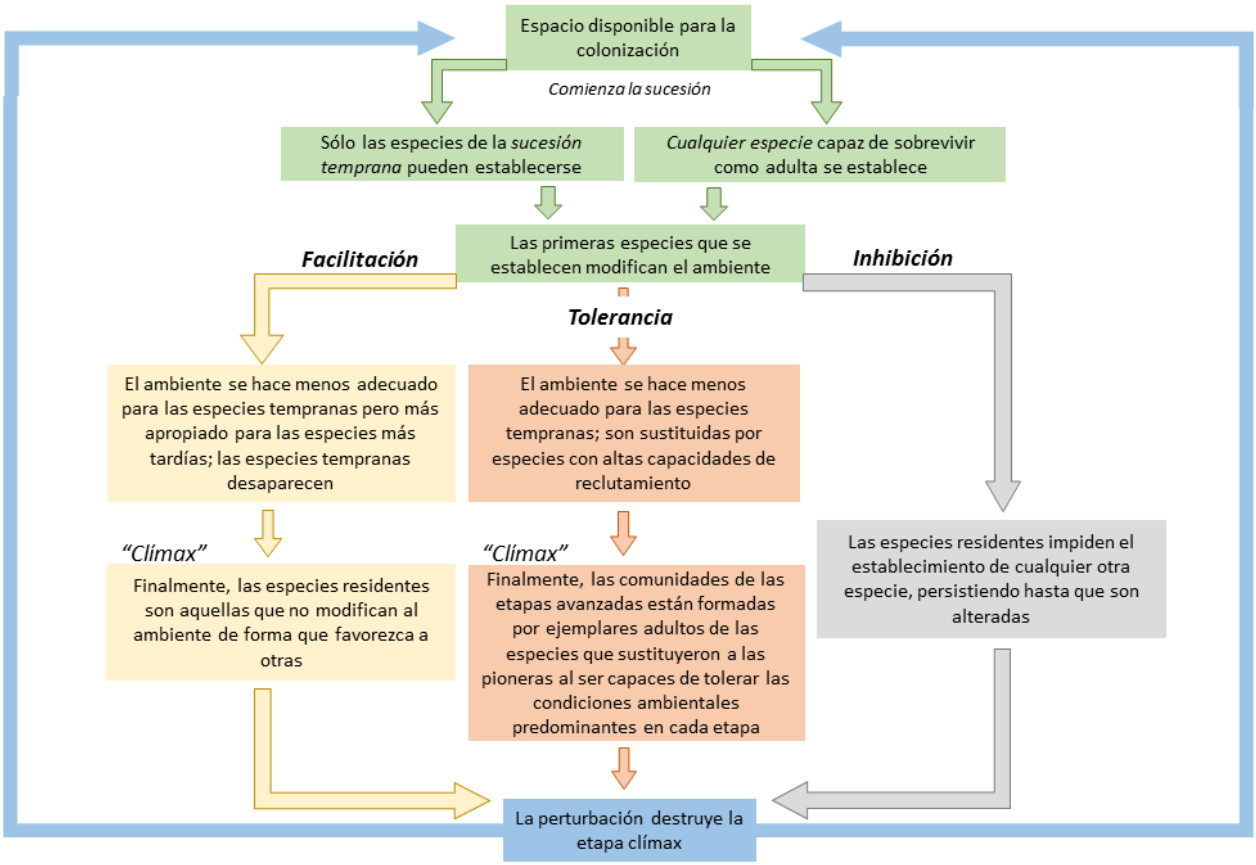


Fig. A.1. Tipos de mecanismos sucesionales y las distintas fases que se producen a lo largo de una sucesión en la que actúe cada uno de ellos. Modificado de Connell y Slatyer (1977).

El mecanismo de tolerancia (naranja en Fig. A1) implica que las especies que se establecen en una zona no la alteran de un modo que favorezca o inhiba las capacidades de asentamiento del resto (Connell y Slatyer 1977; Pickett et al. 1987). De esta manera, las especies que colonizan un medio inicialmente son sustituidas por otras que presentan mejor capacidad de reclutamiento, así como mejores capacidades para competir por los recursos, las cuales pueden permanecer igualmente presentes en etapas intermedias o maduras del desarrollo sucesional (Connell y Slatyer 1977; Farrell

1991). El hecho de que un recambio de especies determinado por un mecanismo de tolerancia sea prácticamente inexistente más allá de las etapas iniciales ha provocado que existan escasos estudios que demuestren su influencia en los procesos de sucesión en suelos (Walker y Chapin 1986; Chapin 1994), limitándose a trabajos centrados en comunidades vegetales de praderas (Rice y Pancholy 1972; Smith y Rice 1983).

Mediante un mecanismo de inhibición (gris en Fig. A1) los organismos asentados en una área modifican las características del medio impidiendo el asentamiento de nuevos colonizadores. La inhibición provoca que sólo sea posible una colonización por organismos diferentes cuando los presentes en la zona pierdan total o parcialmente su posición dominante en el ecosistema, tal y como puede suceder tras procesos de senescencia o tras una perturbación que elimine la mayor parte de los miembros de estas comunidades (Egler 1954; Horn 1981; Pickett et al. 1987; Farrell 1991). Ejemplos de estas interacciones inhibitorias son el secuestro de nutrientes del suelo por diferentes especies de plantas (Kruger 1983; Tilman 1988), el sombreado debido a una alta densidad de las mismas (Walker y Chapin 1986; Tilman 1988; del Moral 1999) o la producción de compuestos alelopáticos de origen vegetal (Tolliver et al. 1995) o antibióticos por parte de microorganismos (Hibbing et al. 2010). A pesar de que se han descrito fenómenos de inhibición para el asentamiento posterior de otras especies en distintos ecosistemas (Seigler 1996; Orr et al. 2005), no se tiene constancia de que sean mecanismos, ni únicos, ni principales, en ninguna sucesión que tenga lugar en el ecosistema edáfico (Matthews 1992).

Por último, el mecanismo de facilitación (amarillo en Fig. A1) implica que ciertas especies que colonizan el medio favorecen que otros organismos diferentes puedan asentarse, mediante la formación de nuevos microambientes y/o la acumulación de recursos. Los nuevos colonizadores pueden desplazar a las especies pioneras al presentar adaptaciones más adecuadas para las nuevas condiciones generadas. Las costras microbianas constituyen un ejemplo de estos mecanismos; los diferentes organismos que las componen colaboran en la formación de una estructura que capta y acumula nutrientes, al tiempo que favorece la creación de unas nuevas condiciones fisicoquímicas, las cuales influyen positivamente en las capacidades de establecimiento posterior de otros organismos (Belnap et al. 2001; Stachowicz 2001; Escudero et al. 2007; Breen y Lévesque 2008; Pointing y Belnap 2012; Schulz et al. 2013). También son

frecuentes los mecanismos de facilitación a nivel de interacciones individuo-individuo, como en el caso de las micorrizas (Allen y Allen 1988; Simard y Durall 2004; van der Heijden y Horton 2009) y en el de las relaciones endosimbióticas entre plantas y procariotas, como entre el género de plantas *Alnus* y el de actinobacterias *Frankia* (Crocker y Major 1955; Torrey 1990; Benson y Silvester 1993; Schwencke y Carú 2001) o entre plantas del género *Gunnera* y cianobacterias del género *Nostoc* (Bonnet y Silvester 1981; Rai et al. 2000; Bergman y Osborne 2002; Guevara et al. 2002; Papaefthimiou et al. 2008). Estas relaciones planta-bacteria están predominantemente presentes en etapas tempranas de la sucesión en sedimentos glaciares, bosques y ambientes alpinos, bien del Hemisferio Norte (Europa y Norteamérica, principalmente) en el caso de *Alnus-Frankia* (Benson y Silvester 1993; Schwencke y Carú 2001; Chaia et al. 2010; Anderson et al. 2013), bien en regiones muy concretas del Hemisferio Norte, como Hawaii, y diversas zonas del Hemisferio Sur, en el caso de *Gunnera-Nostoc* (Bonnet y Silvester 1981; Guevara et al. 2002; Papaefthimiou et al. 2008). En ambos casos, las células microbianas se establecen dentro de las células radiculares de la planta, dónde fijan nitrógeno atmosférico que luego transfieren a las células vegetales, las cuales a cambio les proporcionan compuestos de carbono, predominantemente azúcares (Bonnet y Silvester 1981; Benson y Silvester 1993; Rai et al. 2000; Schwencke y Carú 2001; Chaia et al. 2010; Anderson et al. 2013). Estas relaciones simbióticas son de gran importancia para el ecosistema, ya que permiten a la planta pionera aumentar la captación de nitrógeno y carbono, lo que representa una ventaja en la colonización de los ambientes oligotróficos propios de las estadios poco desarrollados de la sucesión (Reynolds et al. 2003; van der Heijden et al. 2008; Powell y Klironomos 2014). Por otro lado, la simbiosis permite un mayor desarrollo de las comunidades implicadas, provocando una modificación más intensa del sustrato que favorece el asentamiento posterior de otros organismos (Vitousek y Walker 1989; Bergman et al. 1992; Guevara et al. 2002; Densmore 2005; Igual et al. 2006; van der Putten et al. 2013; Powell y Klironomos 2014). Mientras que la importancia de los mecanismos de facilitación para posteriores procesos de colonización, así como la diversidad de las relaciones planta-microorganismo, han sido ampliamente caracterizadas en terrenos colonizados por *Alnus* en simbiosis con *Frankia* (Chapin et al. 1994; Walker y del Moral 2004; Chaia et al.

2010; Anderson et al. 2013), la simbiosis entre *Gunnera* y *Nostoc* ha recibido mucha menos atención (Guevara et al, 2002).

Pese a la posible actuación simultánea de varios mecanismos, la facilitación podría ser el mayoritario cuando hay una alta tasa de recambio de especies, así como en las etapas iniciales de la sucesión, mientras que los mecanismos de tolerancia e inhibición podrían dominar las etapas que presentan una composición de especies más estable, como es el caso de las etapas maduras de la sucesión (Walker y Chapin 1986; Farrel 1991; Bertness y Callaway 1994; Eriksson 2000). Además, se ha observado que una misma especie puede interactuar de manera diferente con diversas especies, dependiendo de las condiciones bióticas y abióticas de la etapa de desarrollo en la que se encuentre (Pickett et al. 1987; Chapin et al. 1994; Fastie et al. 1995; Densmore 2005).

A.2 Comunidades microbianas en el suelo

A.2.1 Desarrollo del suelo

La formación del suelo o pedogénesis es un proceso complejo (Harden 1982; Jenny 1994; Ugolini y Dahlgren 2002; Haugland y Haugland 2008; Mavris et al. 2010) que comienza con la meteorización (mecánica, química y/o biológica) de la roca madre, desarrollándose en paralelo a la sucesión primaria (Crocker y Major 1955; Matthews 1992; He y Tang 2008). A lo largo de este proceso se forman una serie de capas en el suelo, llamadas horizontes, que difieren con la profundidad en composición y textura (Lavahun et al. 1996; Schmidt et al. 2008; Rumpel y Kögel-Knaber 2010; Zumsteg et al. 2011; Jumpponen et al. 2012; Knelman et al. 2012; Schulz et al. 2013). La pedogénesis está condicionada por la interacción entre diferentes factores, tanto abióticos, como bióticos. Como se puede ver en la Fig. A2, en el inicio de la pedogénesis, factores pasivos internos, tales como la topografía, el clima, características fisicoquímicas del sustrato y el *pool* regional de microorganismos, son los más influyentes. Posteriormente, factores dinámicos externos, tales como el grado de erosión, textura, contenido hídrico, cantidad de materia orgánica y nutrientes, junto con la biota que coloniza estos suelos (Fig. A2), adquieren una mayor relevancia en el desarrollo.

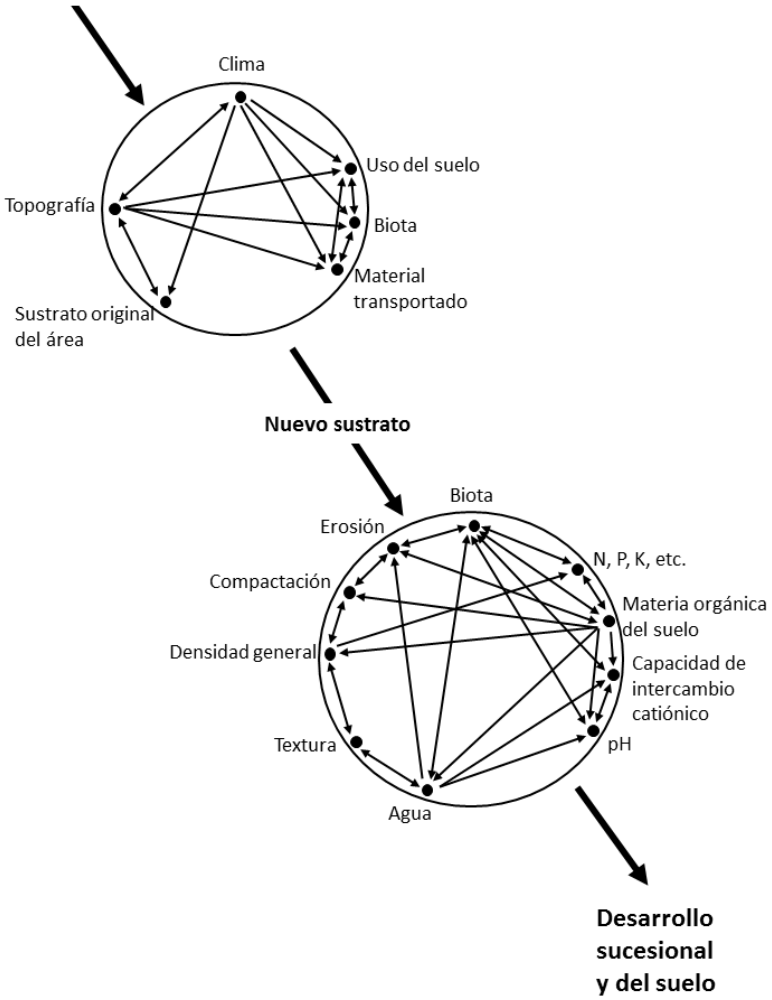


Fig. A.2. Interacciones entre factores que influyen en el desarrollo del suelo. Las flechas exteriores a los círculos indican periodos de tiempo mientras que las internas indican influencias. Modificado de Walker y del Moral (2003).

De entre los factores dinámicos externos, el régimen de precipitaciones es uno de los que se ha considerado más determinante, tanto en las dinámicas de la pedogénesis, como de la sucesión primaria (Ugolini y Dhalgren 2002; Wang et al. 2010; Philippot et al. 2011). En este sentido, la velocidad de desarrollo de un suelo aumenta si las precipitaciones son continuas y moderadas, pudiendo llegar a ralentizarse si alcanzan niveles excesivamente altos, los cuales conduzcan a fenómenos de lixiviación de los nutrientes presentes en el terreno (Ollivier et al. 2011). Por su parte, características estructurales tales como el grado de compactación o la textura de los materiales inertes

del suelo (Fig. A.2) tienen también una gran influencia en la pedogénesis, así como en la colonización microbiana del mismo (Kaiser et al. 1992; Chenu y Stotzy 2002; Schulz et al. 2013). Los microorganismos del suelo, a su vez, influencian la pedogénesis, especialmente en etapas iniciales de desarrollo, mediante acciones químicas y físicas que provocan la disolución de las rocas y del sustrato mineral sobre el que se asientan, en un proceso llamado meteorización biológica (Ascaso y Wierzchos 1996; Gadd 2007; Mapelli et al. 2012). De igual manera, una vez que el desarrollo del suelo permite el establecimiento y crecimiento de plantas, éstas actúan, tanto sobre la estructura edáfica, como sobre la estructura de las comunidades biológicas, ya sea mediante acciones físicas asociadas al desarrollo de sus raíces o debido a los compuestos químicos que éstas exudan, tal y como ha sido descrito en la colonización de áreas descubiertas por retroceso de glaciares (Sigler y Zeyer 2002, 2004; Nemergut et al. 2007; Schmidt et al. 2008; Schulz et al. 2013; Brown y Jumpponen 2014; Ciccazzo et al. 2015).

A.2.2 Diversidad de las comunidades microbianas del suelo

Los microorganismos tienen una gran influencia, tanto en la pedogénesis, como en los procesos de sucesión ecológica en suelos (Schloss y Handelsman 2005; Hamady et al. 2008; Fierer et al. 2010; Simon y Daniel 2011; Nemergut et al. 2013). La mayoría de los conceptos utilizados en la descripción de los procesos sucesionales de comunidades vegetales pueden ser utilizados para estudios similares centrados en las comunidades microbianas (Fierer et al. 2010). Sin embargo, el análisis de las dinámicas de sucesión microbiana conlleva dificultades propias, derivadas principalmente de la imposibilidad de aislar y cultivar la mayor parte de los microorganismos presentes en los suelos, o de la gran influencia que pueden tener incluso pequeñas perturbaciones ambientales sobre las comunidades microbianas (Hill et al. 2000; Fukami y Morin 2003; Kirk et al. 2004; Schloss y Handelsman 2005; Thompson et al. 2005; Schmidt et al. 2008; Monier et al. 2011; Sharma et al. 2012). El desarrollo de técnicas de estudio independientes de cultivo (*e.g.* DGGE, secuenciación masiva, *microarrays*, *etc.*) han permitido superar en parte estas dificultades, ya que permiten caracterizar la estructura completa de la comunidad microbiana, contribuyendo a expandir el conocimiento sobre la composición y la diversidad taxonómica, dinámicas metabólicas, implicaciones

ecosistémicas, *etc.* (Schloss y Handelsman 2005; Hamady et al. 2008; Fierer et al. 2010; Simon y Daniel 2011; Nemergut et al. 2013).

Se estima que las especies de organismos presentes en el suelo pueden representar casi un 25% (1,5 millones aproximadamente) del total de todas las especies de seres vivos descritos en el planeta (Decaëns et al. 2010), aunque la mayor parte de la diversidad podría estar aún sin describir (André et al. 2002; Blaxter et al. 2005, Decaëns et al. 2006). Se ha propuesto que existe una correlación inversa entre el número de especies conocidas de un grupo taxonómico y el tamaño celular o del cuerpo de los organismos del suelo (Decaëns et al. 2006, 2008). Gracias a las nuevas técnicas metagenómicas se ha mostrado que la mayor abundancia y diversidad de los microorganismos edáficos se sitúa en los centímetros más superficiales (Sigler y Zeyer 2002; Fierer et al. 2003; Ekschmitt et al. 2008; Schütte et al. 2010; Zumsteg et al. 2012; Bajerski y Wagner 2013; Brown y Jumpponen 2014; Rime et al. 2015). Sin embargo, las comunidades microbianas presentes a profundidades mayores tienen también una gran influencia en el suelo, tanto en la pedogénesis, como en los procesos de sucesión ecológica (Konopka y Turco 1991; Hiebert y Bennett 1992; Madsen 1995; Richter y Markewitz 1995; Buss et al. 2005; Shen et al. 2010; Eilers et al. 2012; Li et al. 2013). Estos microorganismos presentes en zonas más profundas del suelo tienen adaptaciones fisiológicas diferentes a las que presentan los grupos que se establecen cerca de la superficie, las cuales les permiten colonizar esas áreas (Pedersen y Jacobsen 1993; Vorobyova et al. 1997; Fierer et al. 2003; Rumpel y Kögel-Knaber 2011; Eilers et al. 2012; Rime et al. 2015). La abundancia de bacterias Gram positivas y actinomicetes aumenta con la profundidad, mientras que las bacterias Gram negativas y los hongos disminuyen (Eilers et al. 2012; Frey 2014; Rime et al. 2015), cambios que suceden en paralelo a una disminución importante tanto de los valores de diversidad, como de contenido de biomasa microbiana (Fierer et al. 2003; Zhou et al. 2004; Hartmann et al. 2009; Schulz et al. 2013; Eilers et al. 2012). Esta desigual distribución implica que difieran también las principales actividades de las comunidades microbianas con la profundidad, debido a la citada necesidad del desarrollo de alternativas metabólicas en microorganismos de zonas más profundas para desarrollarse a una menor concentración de nutrientes esenciales (Fierer et al. 2003; Frey et al. 2014).

En el suelo existen numerosos microhábitats asociados a la formación de agregados de fragmentos minerales y materia orgánica (Fig. A.3). Estos agregados permiten la formación de poros que favorecen la aireación o acumulación de agua, así como el desarrollo de comunidades microbianas embebidas en matrices de polímeros extracelulares. La heterogeneidad espacial a nivel de microescala, derivada de la formación de estos agregados, genera una distribución no homogénea de los microorganismos, siguiendo sus requerimientos ambientales específicos (*e.g.* aerobios estrictos, anaerobios facultativos, microaerófilos, anaerobios estrictos, *etc.*) (Swift et al. 1979; Starks et al. 1981; Killham 1994; Atlas y Bartha 2002; Wardle 2002; Decaëns 2010; Vos et al. 2013; Frey et al. 2013).

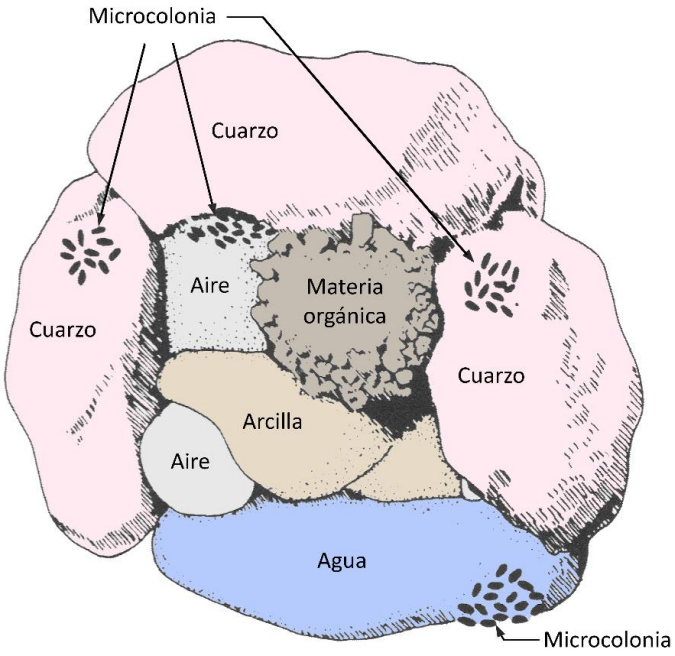


Fig. A.3. Representación de un agregado de suelo que muestra la disposición de las colonias microbianas. Modificado de Brock (1979).

Por otro lado, las condiciones mesoclimáticas del área pueden ejercer también una gran influencia sobre la composición y la diversidad las comunidades microbianas del suelo (Fierer et al. 2003; 2007ab; Gilbert et al. 2010; Nemergut et al. 2007; Noll y Wellinger 2008; Ranjard et al. 2013), así como sobre sus niveles de actividad metabólica (Lombard et al. 2011; Sharma et al. 2012).

En los ecosistemas edáficos existen microorganismos acelulares (virus), microorganismos procariotas (bacterias y arqueas), y microeucariotas (hongos y algas) (Jumpponen et al. 2003; Fierer y Jackson 2006; Nicol et al. 2006; Xu 2006; Fierer et al. 2007b; Seckbach 2007; Srinivasiah et al. 2008; Otto et al. 2010; Bates et al. 2011; Zumsteg et al. 2012; Frey et al. 2013; Tedersoo et al. 2014; Ruggiero et al. 2015). Los virus son microorganismos que carecen de estructura celular, constituidos sólo por material genético (ADN o ARN) protegido por una envuelta proteica, y que únicamente pueden replicarse en el interior de una célula (Breitbart y Rowher 2005; King et al. 2011). Estos microorganismos constituyen el componente biótico más abundante de los ecosistemas terrestres donde pueden llegar a representar entre 3,4 y 4,6 veces el número de bacterias (Breitbart y Rowher 2005; Williamson et al. 2005), siendo más numerosos los que presentan ADN de doble cadena (Srinivasiah et al. 2008). En la actualidad, los virus se clasifican en siete órdenes diferentes y más de 5000 especies (Adams et al. 2015; Krupovic et al. 2016). Aunque su presencia está determinada por la existencia de los hospedadores adecuados en el medio (Rowher 2003; Breitbart y Rowher 2005; Kim et al. 2008; Srinivasiah et al. 2008), factores tales como la composición y la fuerza iónica de las partículas del suelo, el pH, la cantidad de materia orgánica disuelta y el contenido hídrico del suelo influyen también de forma significativa en la presencia de los virus en los ecosistemas edáficos (Williamson et al. 2005). Aunque todavía se conoce poco de la diversidad de este grupo en suelos, sobre todo si se compara con los estudios en ecosistemas acuáticos (Srinivasiah et al. 2008; Zablocki et al. 2014), su importante función como reguladores de las poblaciones de microorganismos, a los que infectan y destruyen en la fase lítica de la infección, hacen de su estudio una pieza clave para entender las dinámicas poblacionales y determinados procesos metabólicos microbianos (Thingstad y Lignell 1997; Kim et al. 2008; Pointing y Belnap 2012; Koskella y Brockhurst 2014).

Las bacterias son considerados los microorganismos más abundantes y diversos del suelo, con hasta 10^9 células y entre 10^3 y 10^6 especies por gramo (Buckley y Schmidt 2002; Torsvik et al. 2002; Gans et al. 2005; Tringe et al. 2005). En la actualidad existen más de 30 filos descritos, según los diferentes volúmenes de la segunda edición del "Bergey's manual of systematic bacteriology" (Garrity et al. 2001; Vos et al. 2009; Krieg et al. 2010; Whitman et al. 2012). Las bacterias son microorganismos procariotas de

pequeño tamaño, con una pared celular compuesta de peptidoglicano en la mayoría grupos. Se diferencia entre bacterias Gram positivas o Gram negativas según la estructura de las paredes celulares (*i.e.* morfología y composición química, *etc*.), tal como revela la tinción diferencial de la pared denominada "Tinción de Gram", desarrollada por el bacteriólogo danés Christian Gram en 1884 (Woese et al. 1990; van Heijenoort 2001; Young 2006; Madigan et al. 2008). Los grupos bacterianos muestran una gran diversidad de morfologías celulares que van desde cocos o bacilos, hasta vibrios y espirilos, pudiendo mostrar o no estructuras de motilidad celular como cilios y/o flagelos. Sin embargo, en la actualidad, los criterios de clasificación de especies se basan principalmente en similitudes de entre el 97 y el 98.7% de la secuencia del gen de la subunidad *16S* del ADNr más que en su morfología (Chun y Rainey 2014; Thompson et al. 2015; Rosselló-Mora y Amann 2015).

Las bacterias presentes en el suelo son capaces de llevar a cabo rutas metabólicas autótrofas y heterótrofas. En el suelo, existe una gran diversidad de compuestos que pueden actuar como donadores de electrones, permitiendo el desarrollo de bacterias, tanto litótrofas, como organótrofas (Bardy et al. 2003; Young 2006; Madigan et al. 2008; Paul 2014). Estas bacterias del suelo pueden encontrarse como organismos de vida libre o estar asociadas a otros seres vivos, ya sea de forma parasítica o simbiótica (Rao et al. 1999; Atlas y Bartha 2002; Barea et al. 2005; van der Heijden et al. 2008; Hayat et al. 2010). Fierer et al. (2007a) diferenció dos grupos en las bacterias del suelo según sus características ecológicas, bacterias copiótrofas y bacterias oligótrofas. Los grupos copiótrofos consumen preferentemente compuestos lábiles de carbono, presentando altos requerimientos nutricionales y elevadas tasas de reproducción, localizadas en zonas con alta disponibilidad de recursos. Por otro lado, las bacterias oligótrofas presentan una alta afinidad hacia sustratos muy específicos, con tasas de reproducción más lentas, y pudiendo desarrollarse de manera más efectiva que las copiótrofas en zonas con baja disponibilidad de nutrientes (McCaig et al. 1999; Marilley y Aragno 1999; Axelrood et al. 2002; Padmanabhan et al. 2003; Fierer et al. 2010).

El dominio *Archaea*, establecido inicialmente por Woese et al. (1990), está compuesto por microorganismos procariotas menos abundantes que las bacterias en la mayoría de los suelos (Auguet et al. 2010), mostrando también una gran variedad de

formas celulares y un tamaño muy similar a las bacterias (Young 2006; Albers y Meyer 2011; Duggin et al. 2015). Las arqueas se diferencian de las bacterias por la ausencia de peptidoglicano en su pared (Albers y Meyer 2011; Koonin y Mulkidjania 2013; Klingl 2014). Poseen, además, determinados genes y rutas metabólicas más similares a los presentes en organismos eucariotas que a los propios de las bacterias (Woese et al 1990; Olsen y Woese 1997; Bernander 2000; Albers y Meyer 2011; Spang et al. 2015). Actualmente, las arqueas se agrupan en un total de 13 *phyla*, con una mayoría de clases incluidas dentro de *Euryarchaeota* (Garrity et al. 2001; Chun y Rainey 2014; Rosselló-Mora 2015; Spang et al. 2015; Zao et al. 2014). Se ha descrito la presencia de arqueas en una gran diversidad de ecosistemas, tanto acuáticos como terrestres (*e.g.* DeLong 1992; Schleper et al. 1997; Ehrhadt et al. 2007; Pointing et al. 2009; Ventosa et al. 2015), más allá de los ecosistemas extremos de los que en un primer momento se las creía exclusivas (Canfield y Raiswell 1999; Auguet et al. 2010), incluyendo áreas de glaciares en retroceso (Nicol et al. 2006), donde llevan a cabo importantes actividades relacionadas con la movilización de compuestos nitrogenados, con el ciclo del metano y con el del azufre, entre otros metabolismos (Canfield y Raiswell 1999; Canfield et al. 2000; Lueders y Friedrich 2000; Nicol et al. 2006; Prosser y Nicol 2008; Offre et al. 2013). Especial interés en suelos ha suscitado el grupo *Crenarchaeota*, debido a su gran abundancia, llegando a representar más del 70% de las secuencias de arqueas analizadas (Ochsenreiter et al. 2003; Auguet et al. 2010; Bates et al. 2011). La disponibilidad de carbono y nitrógeno, junto con el pH del suelo, han sido descritos como los factores principales que influyen en la diversidad, tanto filogenética como metabólica, de las arqueas en el suelo (Bates et al. 2011; Andrew et al. 2012; Offre et al. 2013).

Los hongos constituyen los organismos eucariotas de mayor biomasa en el suelo (Joergensen y Wichern 2008; Baldrian et al. 2012, 2013; Žifcáková et al. 2016), especialmente en áreas boscosas. En estas áreas, en capas superficiales del suelo, se han estimado hasta un máximo de 2000 especies por gramo, valor que varía según si la técnica de diferenciación entre especies se basa en el estudio de la morfología, en el cultivo de los propios hongos, o en el uso de técnicas basadas en diferentes porcentajes de similitud entre secuencias de genes marcadores (O'Brien et al. 2005ab; Buée et al. 2009; Osono y Trofymow 2012; Baldrian et al. 2013; Žifcáková et al. 2016). Los hongos constituyen un linaje monofilético compuesto por organismos quimiorganótrofos,

carentes en su mayoría de flagelos, que presentan una pared celular compuesta fundamentalmente por quitina y quitosano (Free 2013) y el desarrollo de un talo haploide (Blackwell 2011; van der Wal et al. 2013). Pese a ser un grupo de organismos algo menos diverso que las bacterias, consta de aproximadamente 100000 especies descritas, aunque se estima que el número total puede rondar entre los 0,8 y 5,1 millones, agrupadas en 8 *phyla* (Blackwell 2011; Hibbett y Taylor 2013; Tedersoo et al. 2014; Hibbett 2016). Los dos grupos de hongos mayoritariamente presentes en suelos son *Ascomycota* (más de 64000 especies, las cuales incluyen el mayor número de levaduras conocidas) y *Basidiomycota* (más de 32000, incluyendo la gran mayoría de los formadores de micorrizas) (Kirk et al. 2004; van der Wal et al. 2013). Pese a esta diferencia de diversidad entre *Ascomycota* y *Basidiomycota*, según el estudio a escala global de Tedersoo et al. (2014), aproximadamente el 50% del número de secuencias asignadas a grandes grupos taxonómicos de hongos en todo el mundo correspondían a los *Agaricomycetes* (*Basidiomycota*). La saprotrofía, metabolismos relacionados con la movilización de compuestos de carbono y nitrógeno provenientes de la degradación de materia orgánica, parecen ser las rutas metabólicas más comunes en los hongos del suelo (Buée et al. 2009; Damon et al. 2010; Garrett 2013; van der Wal et al. 2013; Žifcáková et al. 2016). También son abundantes aquellos que establecen relaciones simbióticas con otros organismos, como los formadores de líquenes (relaciones con algas y/o cianobacterias, Feuerer y Hawksworth 2007; Lutzoni y Miadlikowska 2009) o los formadores de micorrizas (asociación con plantas vasculares, Oehl et al. 2011; Tedersoo et al. 2014; Thonar et al. 2012). Al igual que para las bacterias, además de las condiciones climáticas, el desarrollo de los hongos en suelos está determinado por otros factores como el *pool* regional de especies de plantas (*i.e.* diversidad y abundancia de las mismas, las características morfológicas y capacidades secretoras de sus sistemas radiculares, *etc.*), o las propiedades fisicoquímicas del suelo como el pH o la concentración de nutrientes tales como carbono, nitrógeno o calcio (Rousk et al. 2010; van der Putten et al. 2010; Tedersoo et al. 2014).

Las algas son un grupo de eucariotas polifilético y heterogéneo caracterizado por su capacidad de realizar la fotosíntesis, pudiendo presentarse como formas unicelulares o pluricelulares (Lund 1967; Nabors 2004; Rasran 2004; Lee 2008). El suelo es el sistema no acuático más importante para las algas, siendo más abundantes y diversas cuanto

mayor sea el contenido hídrico del mismo (Hoffman 1989; Rasran 2004; Zancan et al. 2006; Lee 2008; Wilkinson et al. 2012). Estas algas pueden llegar a encontrarse en concentraciones de hasta 10^8 células por gramo del suelo (Lee 2008), concentrándose principalmente en los milímetros más superficiales del mismo (Atlas y Bartha 2002; Zancan et al. 2006). En zonas más profundas se encuentran mayoritariamente aquellos grupos de algas capaces de llevar a cabo un metabolismo mixotróficos, (*i.e.* heterótrofo facultativo, Rasran 2004; Selosse y Roy 2009; Otto et al. 2010; Figueroa-Martínez et al. 2015). Taxonómicamente, las algas se agrupan en 14 *phyla*, clasificados a su vez un total de 53 clases, compuestas por más de 70000 especies, números que varían enormemente según el método utilizado para describir las poblaciones estudiadas (Guiry 2012). El *phylum Chlorophyta* es el grupo de algas del suelo cuya biología y diversidad han sido más estudiadas (Zancan 2006; Karsten y Holzinger 2014; Wilkinson et al. 2015). Este grupo es común en ecosistemas terrestres de ambientes extremos tales como desiertos (fríos y cálidos) o hábitats hipersalinos (Lewis y Lewis 2005; Schmidt et al. 2011). Algunos grupos son capaces de vivir de manera simbiótica, destacando los fotobiontes de los líquenes (Graham y Wilcox 2000; Lewis y McCourt 2004; Friedl y Bhattacharya 2002). Otros *phyla* de algas capaces de desarrollarse en suelos son *Rhodophyta* (algas rojas), capaces de captar luz a mayores profundidades edáficas que otras algas y de desarrollarse en ecosistemas extremos (Graham y Wilcox 2000; Yoon et al. 2006), y *Ochrophyta* (o *Heterokontophyta*) que agrupa las algas pardas, las *Bacillariophyceae* (diatomeas) y las *Xanthopphyceae*, grupos que tienen una importante presencia en el medio edáfico (Trzcińska y Pawlik-Skowrońska 2008). Especial interés presentan las diatomeas, consideradas como el grupo más diverso de todos los microorganismos eucariotas presentes en el suelo (Vanormelingen et al. 2007; Heger et al. 2012). Las diatomeas son consideradas organismos cruciales en la colonización pionera de suelos desnudos, así como en procesos de sucesión primaria y secundaria, gracias a sus capacidades de introducción de carbono y nitrógeno mediante fotosíntesis y otras actividades de meteorización biológica (Starks et al. 1981; Lukesova 2001; Karsten y Holzinger 2014; Rahmonov et al. 2015).

A.2.3 Actividades metabólicas de los microorganismos del suelo

Los comunidades microbianas del suelo presentan la mayor diversidad metabólica de cuantos ecosistemas se han estudiado hasta la fecha (Wardle et al. 2004). Arqueas, bacterias, hongos y algas participan, a través de su metabolismo, en los ciclos de nutrientes del suelo, teniendo de esta manera un papel relevante en la sucesión ecológica (Schulz et al. 2013; Bradley et al. 2014; Ciccazzo et al. 2015).

A.2.3.1 Ciclo del carbono

En el ciclo del carbono en suelos participan, tanto microorganismos capaces de incorporarlo al suelo por fijación (autótrofos), como aquellos capaces de movilizarlo por degradación de los compuestos presentes en el medio (heterótrofos).

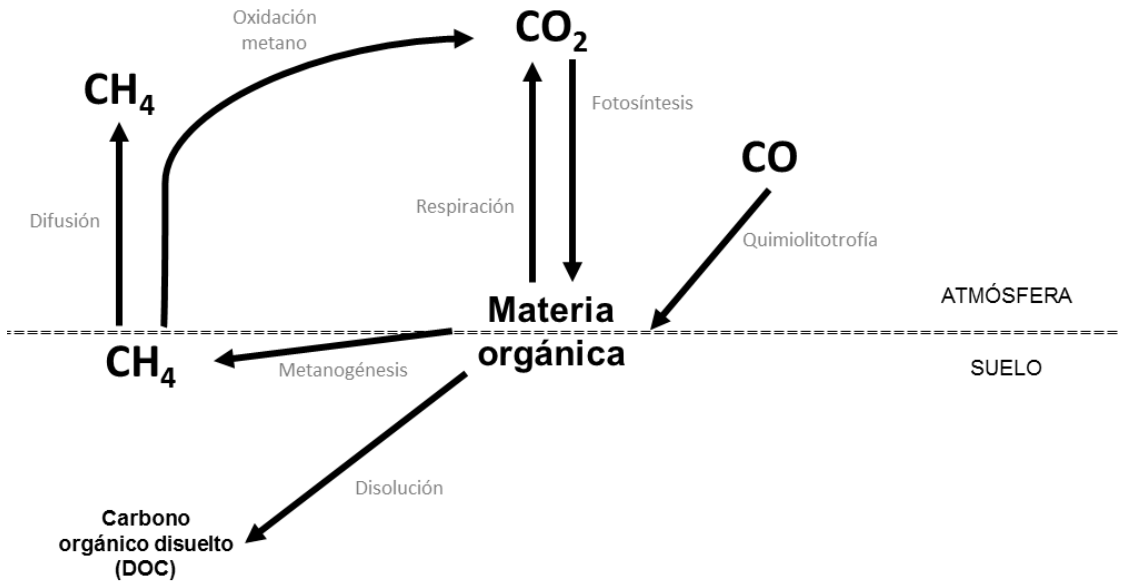


Fig. A.4. Principales actividades (en gris) y compuestos movilizados (en negro) por las comunidades microbianas del suelo dentro del ciclo del carbono.

Los microorganismos fotótrofos, principalmente algas y cianobacterias, participan (junto con las plantas) de la fijación de CO_2 atmosférico mediante la fotosíntesis, situándose este proceso en el centro del ciclo (Fig. A.4, Nemergut et al. 2007; Yergeau et al. 2007; Brankatschk et al. 2011; Bradley et al. 2014). Además, los microorganismos con metabolismos quimiolitótrofos fijan también carbono (en forma

de CO), obteniendo la energía, por su parte, mediante oxidación de compuestos inorgánicos (Tolli y King 2005). El carbono fijado es incorporado a la materia orgánica, la cual es susceptible de ser posteriormente degradada también por acción microbiana, principalmente por bacterias heterótrofas y hongos (Brankatschk et al. 2011; Esperschütz et al. 2011; Tedersoo et al, 2014; Paul et al, 2014). Estos microorganismos heterótrofos son capaces de degradar compuestos orgánicos de muy diferente naturaleza, tanto lábiles como recalcitrantes, ricos en carbono y nitrógeno (Brankatschk et al. 2011, 2013; Hahn et al. 2013). El tipo de compuestos orgánicos acumulados en los suelos, susceptibles de ser degradados por microorganismos, está influido en gran manera por una serie de variables correlacionadas, como pueden ser el contenido en nutrientes que presente el suelo o la presencia y el tipo de vegetación existente en la zona (Ohtonen et al. 1999; Jumpponen et al. 2003; Fierer et al. 2007ab; Guelland et al. 2013; Hahn et al. 2013; Bradley et al. 2014; Brown y Jumpponen 2014; Tedersoo et al. 2014). A través de estas actividades degradativas se produce una pérdida de carbono en estado de gas desde el sistema, mediante procesos que van desde la respiración aerobia, en forma de CO_2, hasta la metanogénesis, en forma de CH_4, resultado de un metabolismo anaerobio (Ekschmitt et al. 2008; Yiqi y Zhou 2010; Nauer et al. 2012; Aronson et al. 2013; Yue et al. 2015). Mientras que la producción de CO_2 mediante respiración está taxonómicamente muy extendida entre la microbiota edáfica (Schlesinger y Andrews 2000; Curiel Yuste et al. 2007; Yergueau et al. 2007; Allison et al. 2010; Yiqi y Zhou 2010; Paul 2014), el metabolismo metanogénico está sólo limitado a algunos grupos de bacterias y arqueas, produciéndose en condiciones anaerobias (Murrel y Jetten 2009). En los ecosistemas edáficos, solo una pequeña fracción del carbono es emitido a la atmósfera en forma de CH_4, ya que una gran parte de este compuesto es transformado a CO_2 gracias a las actividades de las bacterias oxidadoras de metano, presentes en zonas de transición entre microambientes anaerobios y la atmósfera (Fig. A.4, De Vischer et al. 2004; Jia y Conrad 2009).

A.2.3.2 Ciclo del nitrógeno

Las actividades microbianas en suelos tienen una gran importancia dentro del ciclo del nitrógeno, presentando un alto número de genes diferentes implicados en diversas rutas metabólicas (Fig. A.5).

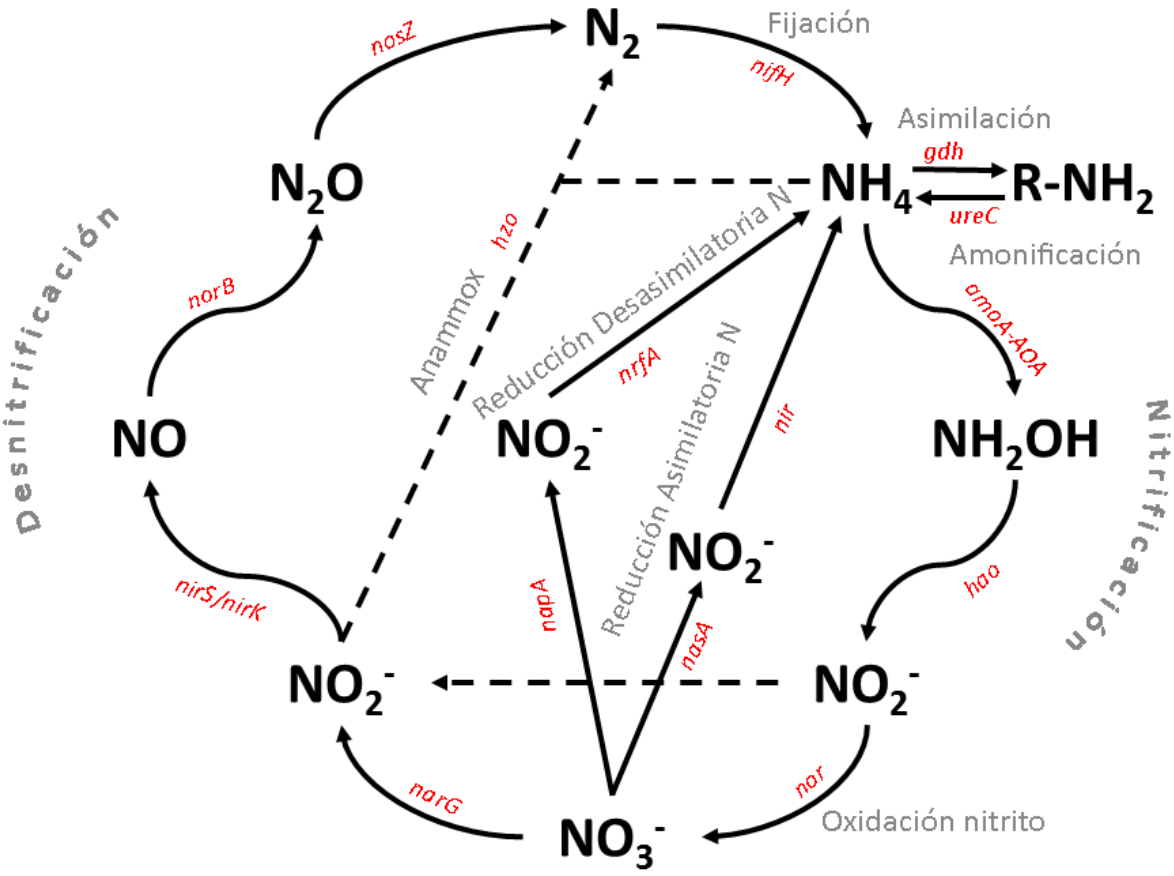


Fig. A.5. Principales actividades (en gris) y compuestos movilizados (en negro) por las comunidades microbianas del suelo dentro del ciclo del nitrógeno. Los genes implicados en cada una de las rutas metabólicas del ciclo aparecen en rojo. En sentido horario: *nifH*, fijación de nitrógeno atmosférico; *gdh*, asimilación de amonio; *ureC*, amonificación; *amoA-AOA*, *hao*, nitrificación; *nor*, oxidación de nitrito; *narG*, *nirS*/*nirK*, *norB*, *nosZ*, desnitrificación; *nasA*, *nir*, reducción asimilatoria de nitrógeno; *napA*, *nrfA*, reducción desasimilatoria de nitrógeno; *hzo*, anammox. Modificado de Zhao et al. (2014).

Determinados grupos de microorganismos, principalmente cianobacterias y proteobacterias, son capaces de fijar el nitrógeno atmosférico mediante la reducción del N_2 a amonio, NH_4^+. Otros son capaces de liberar nitrógeno a través de la degradación

de compuestos orgánicos del suelo ricos en compuestos nitrogenados. Ambos procesos son clave para el funcionamiento de los ecosistemas edáficos, al ser los responsables mayoritarios de la introducción de nitrógeno en las etapas iniciales y transicionales del mismo (Miniaci et al. 2007; Kemmitt et al. 2008; Brankatschk et al. 2011; Ollivier et al. 2011; Ramirez et al. 2012; Arróniz-Crespo et al. 2014; Bradley et al. 2014). El NH_4^+ que resulta de ambas rutas metabólicas puede ser asimilado directamente por los seres vivos, o puede ser oxidado a formas iónicas como nitrito (NO_2^-) y posteriormente nitrato (NO_3^-) vía nitrificación, proceso llevado a cabo mayoritariamente por bacterias nitrificantes quimiolitoautótrofas (Hodson et al. 2008; Ollivier et al. 2011; Levy-Booth 2014; Yu et al. 2014; Ligi et al. 2015). Este NO_2^- generado puede también seguir procesos de desnitrificación, reduciéndose a óxido nítrico (NO), el cual es posteriormente reducido a óxido nitroso (N_2O), generándose tras ello nitrógeno molecular (N_2). Ambos gases suelen difundir del ecosistema edáfico a la atmósfera (Brankatschk et al. 2011; Ollivier et al. 2011; Levy-Booth et al. 2014; Ligi et al. 2015). Existen diversos grupos taxonómicos con capacidad de llevar a cabo procesos de desnitrificación, tanto de bacterias heterotróficas o autotróficas (ambos grupos descritos desde estudios muy iniciales, Baalsrud y Baalsrud 1954; Carlson y Ingraham 1983), como de arqueas (descritas más recientemente, Cabello et al. 2004; Bartossek et al. 2010) o incluso de hongos ascomicotas y basidiomicotas (Bollag y Tung 1972; Shoun et al. 1992). Por otro lado, tanto el NO_2^- como el NO_3^- pueden actuar como aceptores finales de electrones en ausencia de oxígeno, en un proceso llamado reducción desasimilatoria, cuyas rutas metabólicas están principalmente relacionadas con bacterias anaerobias facultativas (Jetten 2008; Zhao et al. 2014; Yue et al. 2015). Un gran número de organismos de diversos grupos (principalmente bacterias, pero también muchas especies de hongos y algas) pueden, por el contrario, incorporar el nitrato en un proceso denominado reducción asimilatoria, metabolismo cuya activación está regulada por la cantidad de amonio extracelular existente en el medio (Atlas y Bartha 2002; Zhao et al. 2014). Además de por fijación atmosférica, el amonio puede generarse gracias a la actividad de un amplio rango de microorganismos que descomponen la materia orgánica, viva o muerta, a través del proceso denominado amonificación (Philippot y Germon 2005; Robertson y Groffman 2014). En contraste a la gran diversidad de organismos nitrificantes, desnitrificantes o amonificadores, solo un reducido grupo de bacterias y

arqueas son capaces de llevar a cabo la oxidación anaerobia de amonio o 'anammox', metabolismo que provoca la pérdida de nitrógeno del medio edáfico al transformar nitrito y amonio a N_2, el cual puede difundir a la atmósfera (Harhangi et al. 2012; Wang et al. 2014).

A.2.3.3 Otras actividades microbianas

Las comunidades microbianas tienen mecanismos de control que pueden influir en su dinámica y desarrollo (Shank y Kolter 2009; Hibbing et al. 2010; Monier et al. 2011; Wei et al. 2015b), los cuales pueden encontrarse también afectando a los procesos de sucesión. Estos microorganismos producen antibióticos, compuestos de diferente naturaleza utilizados en algunos casos como mecanismos de señalización o, mayoritariamente, como sistemas de defensa y control (Keller y Surette 2006; Hoffmann et al. 2007; Yim et al. 2007; Fierer y Lennon 2011). Los antibióticos pueden generarse en situaciones de competencia por recursos y/o espacio (Sigler y Zeyer 2004), producidos para inhibir el crecimiento y actividad de otros grupos competidores (Hibbing et al. 2010), o para promover actividades cooperativas, incluyendo cambios en determinadas rutas metabólicas (Goh et al. 2002; Price-Whelan et al. 2006; Hense et al. 2007). Además, algunos compuestos antibióticos están implicados en respuestas de defensa frente a patógenos (Hibbing et al., 2010; Chan et al., 2013; Koskella and Breitbart, 2014). Por todo ello, la presencia, tanto de estos compuestos en suelos, como la detección de genes microbianos implicados en su producción o en la respuesta a los mismos, denota la existencia de relaciones bióticas interespecíficas en las comunidades microbianas edáficas (Hibbing et al., 2009; Segawa et al. 2013; Wei et al. 2015b).

A.3 Glaciares en retroceso

A.3.1 Distribución y dinámica actual de los glaciares terrestres

Pese a que el número total de glaciares existentes fluctúa entre publicaciones, en la actualidad se acepta que este valor es de aproximadamente 198000, distribuidos en un total de 19 regiones (Fig. A.6, Pfeffer et al. 2014; Field et al. 2014). Quince de estas regiones se encuentran en el Hemisferio Norte y solo cuatro en el Hemisferio Sur (Fig. A.6). La desigual distribución del número de áreas glaciares ha hecho que la mayoría de

los estudios de sucesión en suelos centrados en estas zonas se haya llevado a cabo en el Hemisferio Norte (entre otros, Chapin 1994; Sigler y Zeyer 2002; Wardle y del Moral 2004; Bradley et al. 2014; Brown y Jumpponen 2014), pese a que las zonas con más superficie cubierta por el hielo (aproximadamente 132900 km^2 de extensión actual) sean la Antártida y la región Subantártica (Bliss et al. 2013; Pfeffer et al. 2014). Dentro de esta última, es la región situada en el extremo sur de Sudamérica (número 17 en Fig. A.6) la que presenta un mayor número de zonas glaciares y de superficie cubierta por hielo glaciar (Holmlund y Fuenzalida 1995; Warren y Aniya 1999; Strelin et al. 2008; Masiokas et al. 2009; López et al. 2010; Mouginot y Rignot 2015).

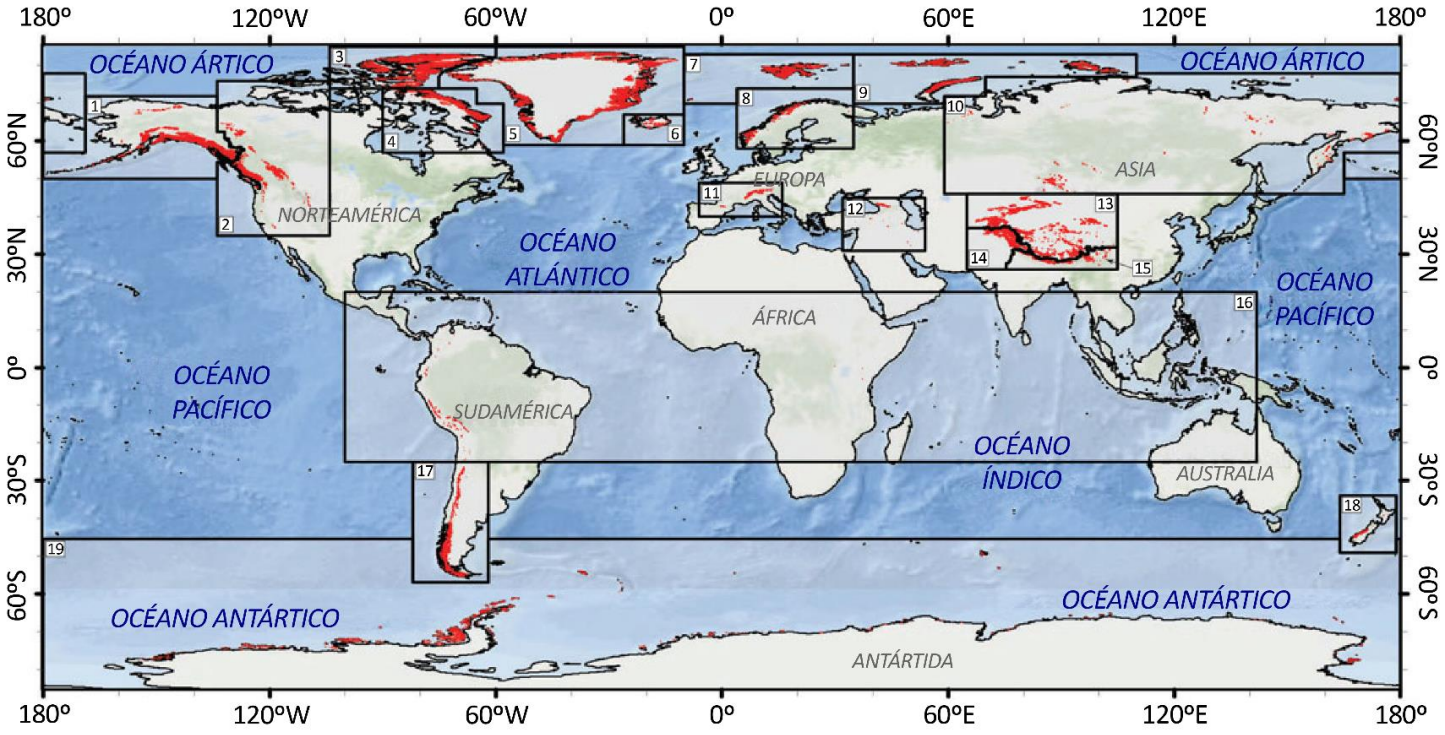


Fig. A.6. Distribución de las regiones glaciares de primer orden según el RGI (recuadradas). Los glaciares se representan en rojo. Modificado de Pfeffer et al. (2014).

En términos generales, los valores actuales de número de glaciares y de superficie total ocupada son menores que los estimados para épocas anteriores, incluso para las zonas subantárticas (Rignot et al. 2003; Rivera et al. 2007; Willis et al. 2012; Abdel Jaber et al. 2014), disminución que viene produciéndose desde mediados del siglo

XIX, coincidiendo con el final del periodo denominado Pequeña Edad del Hielo y el comienzo de la Revolución Industrial (Leclercq et al. 2011; Koch 2015; Zemp et al. 2015). La combinación de factores naturales y antropogénicos es considerada la causa principal de esta pérdida de masa de hielo (Marzeion et al. 2014). La influencia antrópica sobre este proceso ha aumentado considerablemente durante las últimas décadas debido al continuo aumento de la tasa de emisiones de gases de efecto invernadero, conllevando un aumento general de temperatura y la consiguiente aceleración en la pérdida de hielo (Marzeion et al. 2014; Field et al. 2014; Zemp et al. 2015). Sin embargo, las variaciones negativas de extensión y volumen de hielo, observadas en los últimos años en zonas glaciares de todo el planeta (Field et al. 2014; Zemp et al. 2015), son en realidad consecuencia de cambios climáticos ocurridos hace décadas, debido a que las masas de hielo glaciar necesitan, en general, grandes periodos de tiempo para reflejar cambios ocurridos anteriormente en las condiciones climáticas de la zona donde éstas se encuentren localizadas (Marzeion et al. 2014). Este hecho invita a pensar que el calentamiento global acelerado que está teniendo lugar en la actualidad (Field et al. 2014) va a generar procesos de retroceso glaciar mucho más acusados en el futuro cercano.

A.3.2 Estudios de sucesión primaria en cronosecuencias establecidas en el retroceso del glaciar

En zonas de glaciares en retroceso, la retirada de la capa de hielo expone *de novo* superficies terrestres que han estado cubiertas durante periodos prolongados de tiempo, las cuales presentan sustratos de tipología heterogénea (*e.g.* depósitos de arenas, materiales rocosos expuestos, superficies de erosión, llanuras aluviales o incluso lodos, *etc.*) (Lazzaro et al. 2010; Bradley et al. 2014; Rydgren et al. 2014; Ciccazzo et al. 2015). Estos sustratos quedan así accesibles para una posible colonización microbiana, iniciándose entonces un proceso de sucesión primaria (Grubb 1986; Schulz et al. 2013; Bradley et al. 2014; Rydgren et al. 2014). Tras una colonización inicial, llevada a cabo generalmente por microorganismos psicrófilos que pueden estar presentes previamente bajo la capa de hielo o han podido llegar a la zona arrastrados por el agua de escorrentía proveniente de la misma o por deposición eólica (Fierer et al. 2010;

Lazzaro et al. 2010; Frey et al. 2013), se inicia la sucesión, la cual conlleva variaciones en la estructura y composición de las comunidades microbianas y vegetales (Fig. A.7) (Bradley et al. 2014; Ciccazzo et al. 2015; Rime et al. 2015). Las zonas más cercanas al frente glaciar son las que llevan menos tiempo sin estar cubiertas por el hielo y, por tanto, presentan un estado de desarrollo más temprano, mientras que las más alejadas constituyen las etapas más avanzadas de desarrollo sucesional tras un periodo más largo de exposición (Mathews 1992; Chapin et al. 1994; Bardgett 2000; Wardle y del Moral 2003; Nemergut et al. 2007; Walker et al. 2010; Schulz et al. 2013; Rydgren et al. 2014). Esta aproximación, basada en una "sustitución de espacio por tiempo", permite el establecimiento de cronosecuencias para el análisis del desarrollo temporal de un ecosistema sin la necesidad de realizar estudios de parcelas permanentes durante un periodo de tiempo prolongado (Matthews 1992; Walker et al. 2010; Dickie et al. 2013; Phillips 2014).

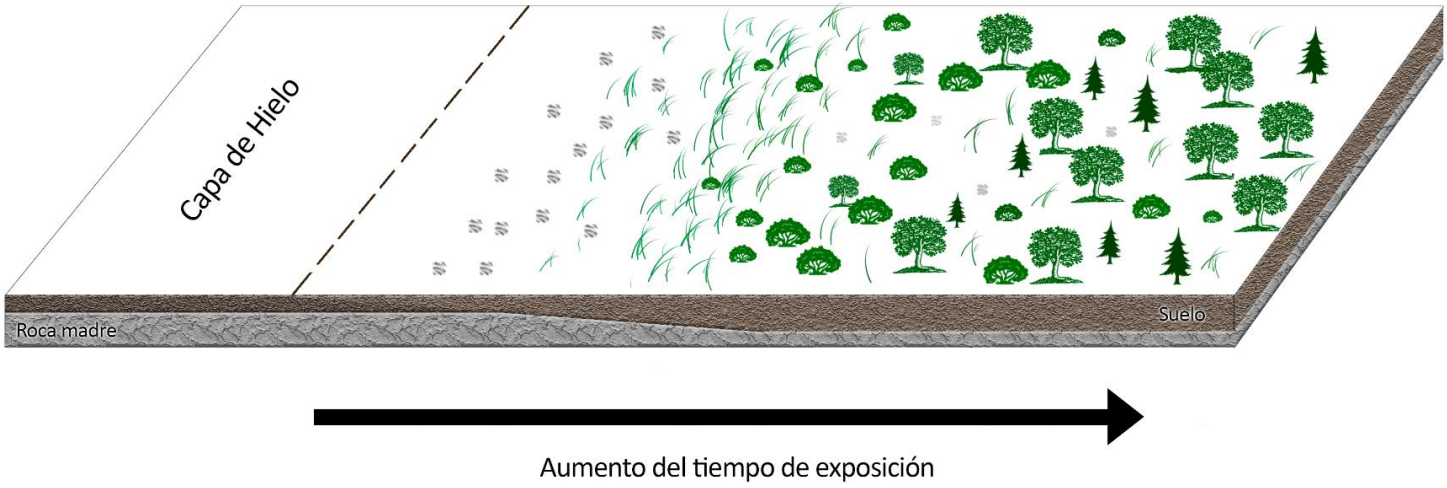


Fig. A.7. Esquema de colonización biológica a lo largo de una cronosecuencia propio de una sucesión iniciada tras el retroceso de un glaciar.

El tiempo que llevan los terrenos glaciares sin estar cubiertos por hielo puede determinarse mediante diferentes metodologías. Una de ellas consiste en el estudio de la edad de las morrenas glaciares que estén presentes, pudiendo utilizarse para ello métodos de datación basados en los isótopos cosmogénicos que presenten los materiales estudiados o en modelos de procesos geomorfológicos (Heyman et al. 2011;

Applegate et al. 2012; Goehring et al. 2012). Un segundo método se basa en la liquenometría, *i.e.* la estimación de la edad de una superficie geológica mediante la medición de la variación del diámetro de líquenes (con una tasa anual de crecimiento conocida) que crecen en bloques rocosos de cierto tamaño presentes en las morrenas (Gordon y Sharp 1983; Armstrong y Bradwell 2010). Otras metodologías ampliamente utilizadas son los estudios de dendrocronología, *i.e.* la datación de la edad de una zona mediante el estudio de los anillos de crecimiento de las plantas leñosas que establecidas en ella, útil solo para las zonas que presentan una vegetación vascular leñosa (Wiles et al. 2011; Schweingruber 2012; Cook y Kairiukstis 2013), así como los basados en imágenes de fotografía aérea de la zona estudiada (Schiefer y Gilbert 2007; Solomina et al. 2016). La combinación de los resultados obtenidos por más de uno de estos métodos permite obtener una información más precisa del tiempo de exposición del terreno estudiado (Evans et al. 1999; Winchester y Harrison 2000; Sancho et al. 2011; Arróniz-Crespo et al. 2014). Además de la estimación del tiempo que llevan los terrenos sin estar cubiertos por el hielo, para poder interpretar correctamente estas cronosecuencias se requiere que las variables ambientales del área donde se establezca la cronosecuencia hayan permanecido relativamente constantes a lo largo del tiempo que abarca el estudio, permitiendo una detección menor de los procesos estocásticos que hayan podido producirse durante el proceso de sucesión (del Moral 2007; Johnson y Miyanishi 2008; Sauer 2010; Walker et al. 2010; Phillips 2014).

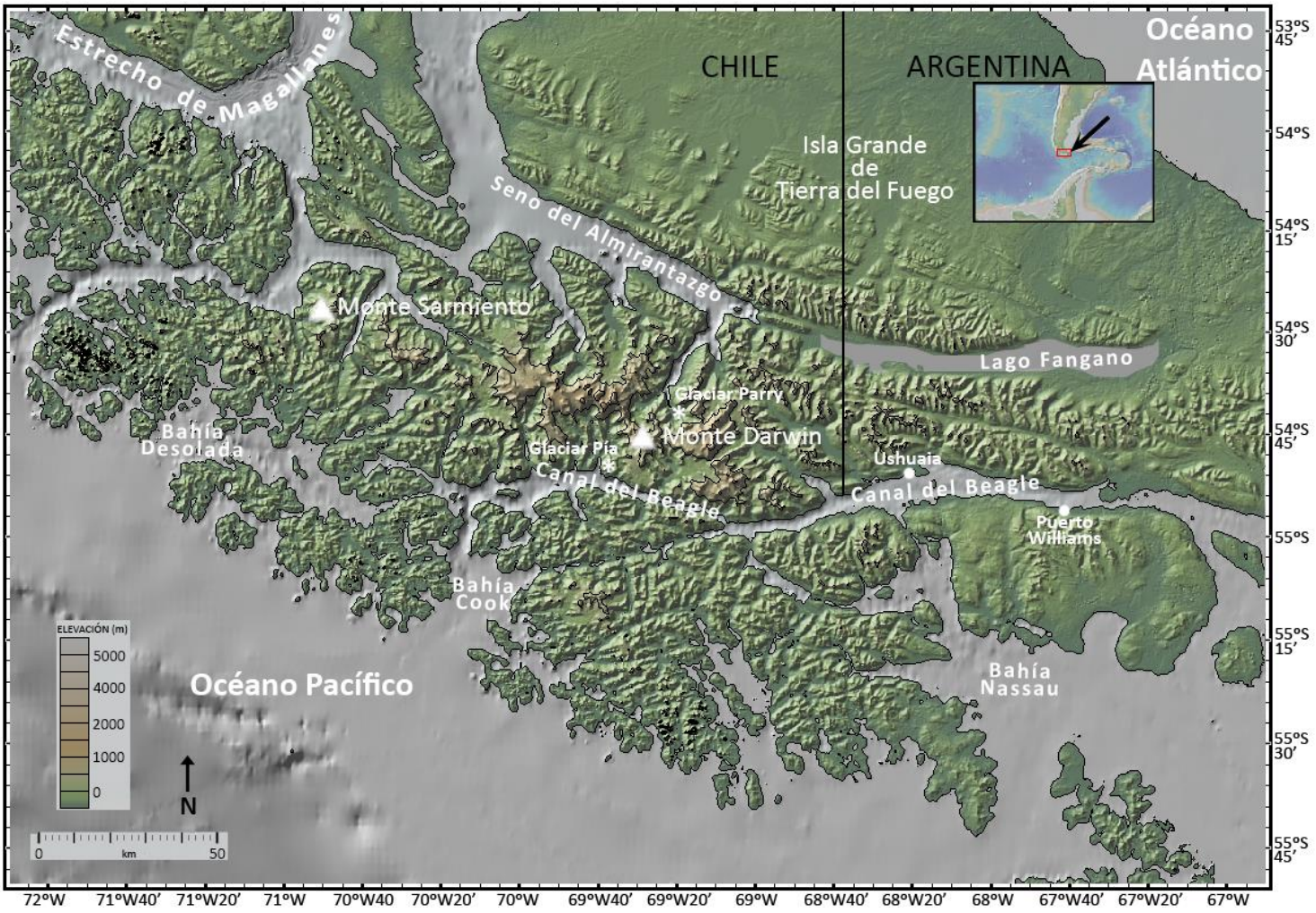
Utilizando el marco de la sustitución del tiempo por espacio se han analizado procesos de sucesión primaria tras el retroceso de glaciares, desde los primeros estudios sucesionales de comunidades vegetales realizados por Muir (1915) y Clements (1916, 1928), hasta otros estudios mucho más recientes (*e.g.* Reiners et al. 1971; Chapin et al. 1994; Walker y del Moral 2003; Wardle et al. 2004; Caccianiga et al. 2006; Walker et al. 2010), como los centrados en comunidades microbianas (*e.g.* Ohtonen et al. 1999; Sigler y Zeyer 2002; Yoshitake et al. 2010; Ciccazzo et al. 2015). En lo referente a la sucesión de comunidades microbianas, estos estudios han abarcado tanto las variaciones taxonómicas que presenta un grupo de microorganismos a lo largo del proceso sucesional (*e.g.* Jumpponen 2003; Nicol et al. 2006; Wu et al. 2012) o distintos grupos simultáneamente (*e.g.* Zumsteg et al. 2011; Schmidt et al. 2014), como los procesos de ensamblaje de las diferentes comunidades microbianas de un medio (*e.g.* Dini-Andreote

et al. 2015; Nemergut et al. 2015), o las variaciones de las mismas en relación a la evolución de las comunidades vegetales (*e.g.* Nara 2006; Blaalid et al. 2012; Knelman et al. 2012; Brown y Jumpponen, 2014). Otros trabajos se han centrado más en las variaciones de las actividades que presentan estas comunidades microbianas a lo largo de la sucesión (*e.g.* Kandeler et al. 2006; Anesio y Laybourn-Parry 2012; Bradley et al. 2014), o han comparado, tanto las variaciones taxonómicas, como metabólicas, entre dos terrenos glaciares cercanos (*e.g.* Sigler y Zeyer 2002; Tscherko et al. 2003; Foght et al. 2004; Skidmore et al. 2005; Philippot et al. 2011; Bajerski y Wagner 2013). Además de la aproximación mediante una cronosecuencia, la mayoría de los estudios centrados en retroceso de glaciares desarrollados en la última década comparten también el haber utilizado metodologías independientes de cultivo, con una incidencia especial de las técnicas metagenómicas en la última década (Bradley et al. 2014; Ciccazzo et al. 2015).

ZONA DE ESTUDIO

B. ZONA DE ESTUDIO

La zona de estudio seleccionada para la realización de esta tesis se localiza en Tierra del Fuego (XII Región de Chile) (Fig. B.1), concretamente las áreas estudiadas con glaciares en retroceso se hayan situadas en la Cordillera Darwin, la cual ha estado cubierta recientemente por hielos glaciares (Masiokas et al. 2009ab). Los estudios fueron realizados sobre muestras de suelo recogidas en dos zonas con evidencia de retroceso glaciar reciente, situadas en la vertiente norte y sur de la Cordillera Darwin, en una expedición que tuvo lugar en diciembre de 2009. En esta expedición se recogieron también ejemplares de la especie herbácea *Gunnera magellanica* en diferentes puntos situado la región de Tierra del Fuego, complementados con muestras recogidas en una campaña de muestreo realizada en enero de 2011.



◄**Fig. B.1.** Mapa de la zona de estudio, localizada en la región chilena de Tierra del Fuego (XII Región). Los terrenos de glaciares estudiados (Glaciar Pía, Glaciar Parry) aparecen marcados con un asterisco, mientras que las zonas donde fueron recogidos los ejemplares de *Gunnera magellanica* aparecen detallados en el capítulo 3 de la presente tesis doctoral. Mapa modificado desde GeoMapApp GMRT (Ryan et al. 2009, http://www.geomapapp.org/).

La Cordillera Darwin es una cadena montañosa que presenta altitudes máximas de *c.* 2500 metros sobre el nivel del mar y que recorre el área de Tierra del Fuego de este a oeste, región que, por el hecho de ser un área remota y estar poco habitada, no ha sufrido acciones antrópicas directas (Koppes et al. 2009; Thébault et al. 2014). El área que ocupa esta cordillera se encuentra limitada al norte por el Seno del Almirantazgo y al sur por el Canal del Beagle (Fig. B.1). El 80% de su superficie está cubierta por hielo, con numerosos glaciares de ladera presentes en las dos vertientes de la cordillera (Fig. B.2, Masiokas et al. 2009a). Estos terrenos helados, incluidos en el llamado Campo de Hielo de la Cordillera Darwin, ocupan una extensión aproximada de 2605 km^2, según mediciones realizadas a lo largo de la última década, lo que los convierte en la tercera región glaciar en superficie de las situadas en zonas de climas templados del Hemisferio Sur (Melkonian et al. 2013). Sin embargo, la extensión cubierta por el hielo glaciar en la Cordillera Darwin se está viendo reducida desde la Pequeña Edad de Hielo (1750-1850 d.C.), especialmente en las últimas décadas, al igual que en las dos áreas glaciares más próximas, los llamados Campos de Hielo Patagónicos Norte y Sur (Rivera et al. 2007; Masiokas et al. 2009ab; López et al. 2010; Davies y Glasser 2012; Willis et al. 2012; Melkonian et al. 2013). Este retroceso glaciar ha sido provocado por progresivos cambios climáticos, como el descenso en las precipitaciones producido durante el siglo XX (Quintana 2004; Masiokas et al. 2009a) y el aumento de la temperatura en la zona, especialmente desde la última década de este pasado siglo (Holmlund y Fuenzalida 1995; López et al. 2010; Masiokas et al. 2009ab).

A

B

Fig. B.2. Glaciar de ladera localizado en la vertiente norte de la Cordillera Darwin (Glaciar Parry). A) Frente glaciar y primeras etapas de la sucesión. B) Terreno totalmente deglaciado (etapas posteriores de sucesión). Fotografías tomadas en diciembre de 2009 por Sergio Pérez-Ortega.

El clima en Tierra del Fuego está caracterizado por la existencia de vientos huracanados, provenientes mayoritariamente del oeste, y de vientos fríos, provenientes principalmente del sur, consecuencia de las marcadas diferencias de presión atmosférica existentes entre las regiones situadas en las latitudes medias del Hemisferio Sur y la Antártida (Oscilación Climática Antártica, 'AAO' en inglés, van Bellen et al. 2015). La región presenta una temperatura media con escasa variación entre zonas (4,5° C en la zona más al sur y 5,9° C en Punta Arenas, unos 200 km al norte) y con muy pocas fluctuaciones a lo largo del año (Santana et al. 2006), habiendo sido incluida por todo lo anterior en la unidad climática subantártica (Morrone 2000; Luebert y Plicoff 2006; Rozzi et al. 2008). Las condiciones atmosféricas, combinadas con la disposición este-oeste de la Cordillera Darwin, los característicos relieves de la misma y la gran influencia del Océano Pacífico generan un marcado efecto orográfico que da origen a una densa y persistente capa de nubes en las áreas de la región de estudio con orientación suroeste (Holmlund y Fuenzalida 1995; Strelin e Iturraspe 2007; Koppes et al, 2009; López et al, 2010). Esta combinación de factores provoca, a su vez, un marcado gradiente climatológico a lo largo de la Cordillera Darwin, especialmente significativo en lo referente a valores de precipitación. El volumen de estas precipitaciones es creciente desde la costa del Océano Atlántico hacia la del Océano Pacífico (con una diferencia de aproximadamente 3500 mm de precipitaciones anuales entre los puntos más al este y más al oeste de los estudiados en esta tesis), presentando además una diferencia de aproximadamente el doble entre la vertiente norte (*c.* 800 mm anuales) y la sur (*c.* 1600mm anales) de la Cordillera Darwin (Holmlund y Fuenzalida 1995; Koppes et al. 2009).

La roca madre en esta zona está formada por granito monolítico sin fracturar, por lo que los suelos no albergan fragmentos minerales macroscópicos provenientes de la misma, presentando un límite bien definido entre los horizontes más profundos y el lecho de roca, el cual se encuentra a una profundidad de entre unos pocos centímetros en las zonas de suelo desnudo y hasta 60 cm en zonas boscosas (Thébault et al. 2014). La vegetación se enmarca dentro de un bioclima antiboreal hiperoceánico, el cuál presenta ombrotipos húmedos e hiperhúmedos (Luebert y Plicoff 2006). En las zonas recientemente deglaciadas, los terrenos se caracterizan por una abundante presencia de líquenes y briófitos (Sancho et al. 2011; Arróniz-Crespo et al. 2014) y por la temprana

colonización por parte de ejemplares de *G. magellanica*, tras la cual el ecosistema avanza hacia fases arbustivas, caracterizadas por la presencia de *Empetrum rubrum* y *Gaulteria mucronata*, hasta fases boscosas dominadas por ejemplares maduros de diferentes especies de árboles del género *Nothofagus*, especialmente *N. betuloides* (cohigue) y *N. pumilio* (lenga) con presencia de *N. antarctica* (ñirre) y *Drimys winteri* (canelo) (Fig. B.3, Sancho et al. 2011; Thébault et al. 2014).

Fig. B.3. Distintas fases de colonización vegetal en terrenos de retroceso glaciar en Tierra del Fuego. A) Colonización liquénica y por *Gunnera magellanica* (asterisco), característica de etapas iniciales de la sucesión. B) Colonización arbustiva, junto con ejemplares jóvenes de *Nothofagus* (asterisco), característica de etapas intermedias de la sucesión. C) Fase boscosa de colonización vegetal, característica de etapas avanzadas de la sucesión. Fotografías tomadas en diciembre de 2009 por Sergio Pérez-Ortega.

El desarrollo de las comunidades vegetales y la formación de los bosques de *Nothofagus* es diferente en las dos vertientes de la Cordillera Darwin, siendo este proceso más rápido en la vertiente sur de la cadena montañosa (Sancho et al, 2011; Arróniz-Crespo et al, 2014) (Fig.B.4). En ambas vertientes, la colonización vegetal se inicia con plantas herbáceas, generalmente de la especie *Gunnera magellanica*, tras aproximadamente 7 años en la vertiente sur y tras 20 en la vertiente norte (Fig. B.4). La colonización arbustiva empieza tras algo más de 10 años en la vertiente sur y más de 26 en la norte, mientas que el desarrollo de los bosques se inicia tras 19 años de exposición del suelo en la vertiente sur y casi 66 en la norte (Fig. B.4).

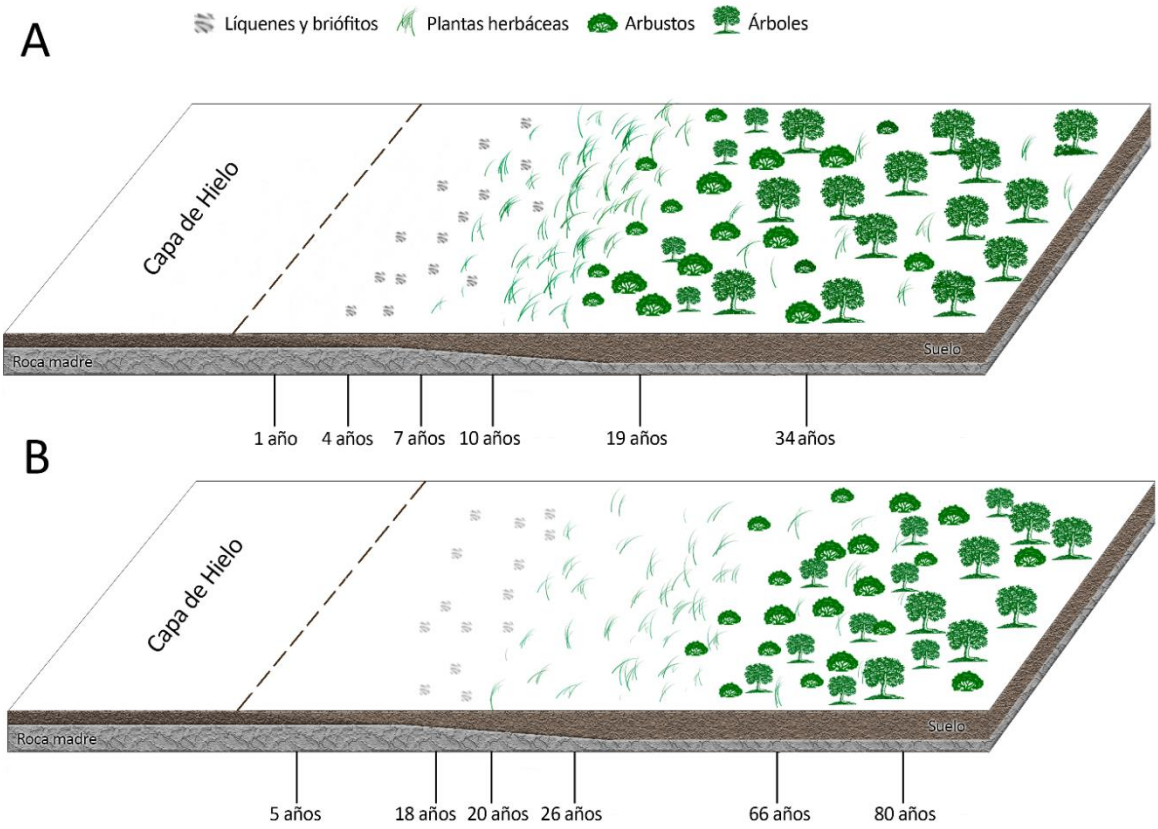


Fig. B.4. Esquema de las cronosecuencias de colonización en terrenos de retroceso glaciar en Tierra del Fuego. A) Cronosecuencia de la sucesión en el Glaciar Pía (vertiente sur de la Cordillera Darwin). B) Cronosecuencia de la sucesión en el Glaciar Parry (vertiente norte de la Cordillera Darwin).

OBJETIVOS Y ESTRUCTURA DE LA TESIS

C. OBJETIVOS Y ESTRUCTURA DE LA TESIS

El objetivo general de la presente tesis doctoral es el estudio de la colonización microbiana en suelos descubiertos tras el retroceso de glaciares en Tierra del Fuego y la caracterización de la sucesión primaria asociada en estas áreas.

Para lograr este objetivo general se propusieron los siguientes objetivos específicos.

- Caracterización de la diversidad taxonómica y funcional de las comunidades microbianas en distintas etapas de sucesión, a lo largo de cronosecuencias establecidas en suelos descubiertos por el retroceso de glaciares en Tierra del Fuego.
- Comparación de las dinámicas de recambio sucesional de las comunidades microbianas del suelo tras el retroceso de dos glaciares de Tierra del Fuego, situados en zonas próximas, pero que difieren en las condiciones macroclimáticas.
- Descripción de la prevalencia de diferentes grupos taxonómicos en las distintas etapas de sucesión en áreas deglaciadas en Tierra del Fuego y de las asociaciones entre los mismos a lo largo de las cronosecuencias.
- Análisis de la diversidad de microsimbiontes asociados a *Gunnera magellanica*, una planta clave en la colonización de áreas descubiertas por retroceso de glaciares en Tierra del Fuego.

La consecución de los objetivos planteados se realizó a través de tres estudios complementarios, presentados como tres capítulos diferentes, que corresponden a tres trabajos publicados o en proceso de revisión en revistas científicas internacionales. Una discusión global final intenta ofrecer una visión completa de los procesos de colonización y sucesión de las comunidades microbianas de los suelos en glaciares en retroceso de la región chilena de Tierra del Fuego.

Capítulo 1: Dinámicas de sucesión microbiana a lo largo de cronosecuencias establecidas en zonas de glaciares en retroceso de Tierra del Fuego (Chile).

Título del artículo científico enviado: "Microbial succession dynamics along glacier forefield chronosequences in Tierra del Fuego (Chile)".

Autores del artículo científico enviado: Miguel Ángel Fernández-Martínez, Sergio Pérez-Ortega, Stephen B. Pointing, Leopoldo G. Sancho y Asunción de los Ríos.

Artículo enviado a la revista *Polar Biology*.

Este primer capítulo recoge el estudio del ensamblaje de las comunidades microbianas del suelo (composición y recambio de especies) tras el proceso de colonización inicial, y a lo largo de cronosecuencias establecidas en las áreas de retroceso de dos glaciares de la Cordillera Darwin (Pía y Parry), con distintas dinámicas de sucesión vegetal y condiciones macroclimáticas. La hipótesis de partida es que a partir de comunidades microbianas similares en ambos glaciares al inicio de la sucesión, habrá una evolución diferente en cada uno de ellos, de manera similar a lo observado para las dinámicas sucesionales de las comunidades vegetales. Para caracterizar la diversidad existente en las comunidades microbianas edáficas se realizó la secuenciación masiva mediante pirosecuenciación 454 de fragmentos *barcoding* del ADN genómico. En concreto, para bacterias se amplificó y secuenció la región V1-V3 de la subunidad *16S* del ADNr, para hongos las regiones 1 y 2 del espaciador interno transcrito (ITS) junto con el gen *5.8S* de ADNr, y para algas se amplificó la región *rbc*L del gen de RuBisCO.

Capítulo 2: Ecología funcional de las comunidades microbianas del suelo a lo largo de zonas de retroceso de un glaciar en Tierra del Fuego (Chile).

Título del artículo científico enviado: "Functional ecology of soil microbial communities along a glacier forefield in Tierra del Fuego (Chile)".

Autores del artículo científico enviado: Miguel Ángel Fernández-Martínez, Stephen B. Pointing, Sergio Pérez-Ortega, María Arróniz-Crespo, T. G. Allan Green, Leopoldo G. Sancho y Asunción de los Ríos.

Artículo enviado a la revista *Frontiers in Microbiology*.

Este segundo capítulo aborda el análisis de la estructura funcional de las comunidades microbianas a lo largo de la cronosecuencia establecida en los terrenos deglaciados del Glaciar Pía. La hipótesis inicial es que la rápida variación de la estructura de las comunidades microbianas y vegetales a lo largo de la cronosecuencia está asociada a diferencias en la estructura funcional de los microorganismos, en especial en actividades relacionadas con el ciclo del carbono y el nitrógeno. El análisis del potencial funcional de las comunidades microbianas presentes en las distintas etapas sucesionales se analizó con el *microarray* GeoChip 4.0. Está metodología está basada en la hibridación del ADN genómico de las comunidades microbianas, previamente extraído y marcado, con una colección de sondas de oligonucleótidos correspondientes a secuencias de distintos genes representativos de rutas metabólicas, presentes en grupos taxonómicos diferentes. Concretamente, en este estudio nos hemos centrado en los genes correspondientes a rutas metabólicas del ciclo del carbono y del nitrógeno, así como los genes implicados en respuestas a condiciones de stress, de resistencia a antibióticos y marcadores de diversidad de virus, por ser todos ellos considerados como factores muy importantes a la hora de estructurar las poblaciones microbianas y, por tanto, determinar su evolución.

Capítulo 3: Diversidad de los *Nostoc* endosimbiontes de *Gunnera magellanica* en Tierra del Fuego, Chile.

Título del artículo científico publicado: "Diversity of endosymbiotic *Nostoc* in *Gunnera magellanica* from Tierra del Fuego, Chile".

Autores del artículo científico publicado: Miguel Ángel Fernández-Martínez, Asunción de los Ríos, Leopoldo G. Sancho y Sergio Pérez-Ortega.

Artículo publicado en la revista *Microbial Ecology*, nº 66, pp. 335-350, 2013.

Este tercer capítulo presenta un estudio de la simbiosis de cianobacterias con la planta herbácea *Gunnera magellanica*, muy común en Tierra del Fuego donde actúa como colonizador pionero. La presencia de cianobacterias simbiontes del género *Nostoc* en los rizomas de esta planta le confiere ventajas competitivas frente a otras especies vegetales, especialmente en suelos con escasa disponibilidad de nutrientes. En este

trabajo estudiamos las relaciones filogenéticas de los microsimbiontes, así como la variabilidad genética de los mismos a varias escalas anidadas (*i.e.* individual, poblacional y regional), en áreas situadas al sur y al norte de la Cordillera Darwin y zonas recientemente deglaciadas o con procesos de sucesión secundaria. Para ello, se amplificaron y secuenciaron parte del gen de la subunidad *16S* del ADNr, el espaciador interno transcrito (ITS, entre los genes *16S* y *23S* de ADNr) y el gen que codifica para la subunidad grande de la enzima RuBisCO unida a la secuencia de su promotor y a la de unas proteínas semejantes a chaperonas (región *rbc*LX) de las cianobacterias presentes en las células vegetales infectadas del tejido radicular de la planta. Estos resultados fueron objeto de análisis de la diversidad haplotípica que presentaban. Además, se caracterizó ultraestructuralmente estas asociaciones simbióticas mediante microscopía electrónica de barrido a baja temperatura (LTSEM), técnica que permite analizar muestras hidratadas y obtener una buena visualización de la interacción entre células hospedadoras y microsimbiontes y de las matrices de exopolisacáridos asociados a las mismas.

CAPÍTULOS

"La vida solo puede ser comprendida mirando hacia atrás, pero ha de ser vivida mirando hacia adelante"

Sören Aabye Kierkgaard

CAPÍTULO 1

Dinámicas de sucesión microbiana a lo largo de cronosecuencias establecidas en zonas de glaciares en retroceso de Tierra del Fuego (Chile)

Resumen

Este estudio examinó la sucesión primaria en comunidades de bacterias, hongos y algas de dos terrenos deglaciados situados en vertientes opuestas de la Cordillera Darwin (Tierra del Fuego, Chile). Las cronosecuencias establecidas en el suelo de las vertientes sur y norte han permanecido sin estar cubiertas por el hielo un número variable de años (entre 1 y 34 y entre 5 y 66 respectivamente), mostrando además factores climáticos y tasas de sucesión de plantas contrastados. Una aproximación mediante secuenciación masiva reveló que entre las bacterias, las cianobacterias dominaban los suelos más jóvenes situados cerca del frente de hielo del glaciar, mientras que las alfaproteobacterias y las acidobacterias incrementan su abundancia con la edad de la superficie del suelo. Hongos formadores de líquenes y parásitos fueron los grupos fúngicos más abundantes en las etapas iniciales de la sucesión, mientras que órdenes de hongos saprófitos y micorrícicos dominaron las etapas de sucesión más avanzadas. El orden *Prasiolales*, por su parte, dominaba las comunidades de algas situadas cerca del frente de hielo del glaciar, mientras que los órdenes *Microthamniales* y *Chlamydomonadales* dominaban las siguientes etapas de la sucesión. Nuestras observaciones reflejan una estructura de la comunidad que cambia con el tiempo para los tres grupos examinados, procesos relacionados con el recambio de taxones en ellos durante la sucesión. El análisis simultáneo de comunidades de bacterias, hongos y algas resalta las diferentes trayectorias mostradas por cada uno de los tres grupos, con patrones de sucesión más señalados para las comunidades bacteriana y fúngica. Se detectó que ambos terrenos deglaciados diferían en las dinámicas temporales, indicando que los factores climáticos, además de afectar a las tasas de sucesión de las plantas, afectan también a las tasas de recambio de las comunidades microbianas y, por tanto, a sus procesos de sucesión primaria.

Abstract

This study examined primary successions of bacteria, fungi and algae in two glacier forefields on opposite slopes of the Cordillera Darwin (Tierra del Fuego, Chile). Southern and northern slope soil chronosequences have been ice-free for varying numbers of years (1-34 and 5-66 years respectively) and show contrasting climate

factors and plant succession rates. A high-throughput sequencing approach revealed that among the bacteria, *Cyanobacteria* were found to dominate younger soils close to the glacier terminus, while abundances of *Alphaproteobacteria* and *Acidobacteria* increased with soil surface age. Lichen-forming and parasitic fungi were the most abundant fungal groups in younger succession stages, whereas saprophytic and mycorrhizal orders dominated later ones. The order *Prasiolales* dominated algal communities close to the glacier terminus, while *Microthamniales* and *Chlamydomonadales* orders dominated subsequent succession stages. Our observations reflect a changing community structure over time for the three microbial groups examined, and these were associated with replacement of taxa during the succession. Our simultaneous analysis of bacterial, fungal and algal communities highlights the different trajectories of the three groups, with more marked succession patterns for the bacterial and fungal communities. Different temporal dynamics were detected for both glacier forefields, indicating that climate factors, besides affecting plant succession rates, affect the rate of microbial community assembly and, consequently, their primary succession.

1.1 Introduction

Signs of global warming have been detected in almost every region of the world, but perhaps the most visible indicators of the effects of climate change are the melting of glaciers and ice caps (Field et al. 2014). Once a glacier has retreated, previously ice-covered soil and rocks are exposed, thus permitting new microbial colonization. Microbial communities that settle after ice retreat may be formed by psychrophilic or psychrotolerant microorganisms present in subglacial sediments or ice sheets and those that colonize the soil just after its exposure (Foght et al. 2004; Hodson et al. 2008; Takeuchi 2011). This initial colonization plays an important role in primary succession, as it enables later colonization by other microorganisms, lichens, bryophytes and vascular plants, and this introduces nutrients into the ecosystem (Grubb 1986; Frenot et al. 1995; Frenot et al. 1998).

In retreating glaciers, the temporal dynamics of microbial and plant colonization and soil development can be investigated through the analysis of chronosequences along glacier forefields (Bardgett and Walker 2004; Knelman et al. 2012; Brown and Jumpponen 2014; Welc et al. 2014; Ciccazzo et al. 2015). In chronosequences, distance to the current glacier terminus serves as a proxy for time since glacier ice retreat (Matthews 1992; Chapin et al. 1994; Bardgett 2000; Sigler et al. 2002; Jumpponen 2003; Bardgett and Walker 2004; Nemergut et al 2007; Walker et al. 2010). Along this chronosequence, soil develops from sand-like sediment in the proximity of the glacier terminus to well-differentiated, physically and chemically complex deposits at the final stages of the succession (Matthews 1992). Interaction between soil structure and microbial community composition is reciprocal insofar as soil structure determines microbial assemblage composition and microbial activities influence soil properties (Bardgett et al. 2005; Bowker et al. 2014; Phillips 2014; Rime et al. 2015). In the initial stages of soil development, bacteria and fungi are key components of the ecosystem, as they are able to mobilize nutrients (*e.g.* via rock bioweathering and carbon and nitrogen fixation processes) facilitating their use by other organisms (Hodkinson et al. 2003; Nemergut et al. 2007; Gorbushina and Broughton 2009; Zumsteg et al. 2012). Algae also play an important role in these initial, non-vegetated stages, due to their capacity to fix carbon (Kastovská et al. 2005; Frey et al. 2013). Plant succession begins as soon as the soil is able to support their growth, which significantly affects the soil attributes (Chapin

et al. 1994; Walker et al. 2003; Bryant et al. 2008; Knelman et al. 2012; Brown and Jumpponen 2014). Abiotic factors (*e.g.* precipitation, sunlight exposure, soil pH, soil organic compounds) can also contribute to soil development and shaping of its community structure (Sigler and Zeyer 2002; Wang et al. 2010; Wu et al. 2012). However, it is still poorly understood how differences in abiotic parameters affect the dynamics of primary succession in glacier forefields, because comparative studies between glaciers are scarce (Sigler and Zeyer 2002; Tscherko et al 2003).

By examining changes in the composition of different microbial groups with increasing soil surface age, insight can be gained into microbial assembly dynamics. In addition, through the simultaneous analyses of the succession dynamics of different taxonomic groups it is possible to recognize the interacting microbial patterns in soils after glacial retreat. Bacterial and fungal succession dynamics have been recently compared across glacier forefields using high throughput molecular sequencing technologies (Brown and Jumpponen 2014; Rime et al. 2015). However, despite the fact that algae are important functional components of initial succession stages (Nemergut et al. 2007), according to our knowledge, no study has simultaneously characterized bacterial, fungal and algal successions in glacier forefields.

The succession dynamics of microbial communities has been investigated in detail in glacier forefields of the Northern Hemisphere (Wang et al. 2010; Zumsteg et al. 2012; Bradley et al. 2014; Brown and Jumpponen 2014), yet little information exists for glaciers in temperate and subpolar regions of the Southern Hemisphere. The present study focuses on Cordillera Darwin, in the southwestern region of Tierra del Fuego (Chile), where glacier retreat has been occurring since the XIX century (Masiokas et al. 2009) and quick succession to stages dominated by *Nothofagus* tree species has taken place (Sancho et al. 2011). The southern and northern slopes of the Cordillera Darwin are sensitive to climate factors. Thus, higher precipitations are recorded for the southern slope facing the Beagle Channel than for the northern side (Holmlund and Fuenzalida 1995).

This study was designed to compare microbial succession patterns and community assembly dynamics across two glacier forefields on different slopes of Cordillera Darwin, consequently presenting different climate conditions. The regional pool of colonizer microorganisms is assumed to be similar as the glaciers are only a few

kilometres apart. However, different dynamics of plant succession and soil development after ice retreat have been observed (Sancho et al. 2011; Arróniz-Crespo et al. 2014). We hypothesized that the different climate conditions (mainly precipitation regime) on both sides of the Cordillera Darwin also affect microbial colonization dynamics and community assemblage. To this end, algal, fungal and bacterial compositions were explored at sites corresponding to different succession stages of two glacier forefields.

1.2 Materials and Methods

Study area

The study area included two glacier forefields located approximately 15 km apart on the southern and northern slopes of Cordillera Darwin, in southwestern Tierra del Fuego (XII Region, Chile, South America). Around 80% of this rugged landscape is covered by an ice cap and glacier outlets flow towards the sea on both slopes. These glaciers have been receding constantly since the Little Ice Age (around 1750 to 1850 AD) (Masiokas et al. 2009) at different speeds and a clear sequence exists of moraine bands (Sancho et al. 2011; Arróniz-Crespo et al. 2014). The southern slope glacier forefield (hereafter designated SS), Pia Glacier (54° 46’ S 69° 40’ W), faces the east arm of the Beagle Channel (Bahia Pia). This slope shows a biological succession that spans 34 years starting with bare soils and ending with a full *Nothofagus* spp. forest (Sancho et al. 2011). The northern slope glacier forefield (hereafter designated NS), Parry Glacier (54° 41’S 69° 23’ W), is bounded towards the north by the Almirantazgo Fjord (Seno Almirantazgo). This slope shows a much slower plant succession than Pia Glacier, with comparable full forest stages observed at sites with soil surface ages higher than 66 years (Holmlund and Fuenzalida 1995; López et al. 2010; Arróniz-Crespo et al. 2014). The Cordillera Darwin area has a maritime climate along the coast characterized by hurricane-force winds (strongest from the West, coldest from the South), dense cloud cover (especially over the southern and western fjords of the study area) and an average temperature of 5°C, with little seasonal variation (Molina 1983; Burgos 1985; Santana et al. 2006). These features, combined with the influence of the Pacific Ocean, create a noticeable SW-NE precipitation gradient. Specifically, rainfall on southern slopes (SS) of

the Cordillera Darwin is twofold (c. 1600 mm per year) that recorded for northern slopes (NS, *c.* 800 mm per year) (Holmlund and Fuenzalida 1995; Koppes et al. 2009).

Sampling

Soil samples were collected in December 2009 across chronosequences dated according to aerial photographs, dendrochronology (tree rings sampling) and lichenometry (Sancho et al. 2011; Arróniz-Crespo et al. 2014). Across each chronosequence, sites that have been ice-free for different number of years, corresponding to different succession stages, were examined (Table 1.1): six in SS (1, 4, 7, 10, 19 and 34 years of soil exposure) and five in NS (5, 18, 20, 26 and 66 years of soil exposure). At each site, 3 sampling points were selected along 3 parallel transects established in each glacier forefield, from the glacier terminus towards the oldest dated moraines. At each sampling point, we obtained composite samples (*c*. 200 g) comprised of 3 subsamples collected within a *c.* 1 m distance. Only the top centimetres (0-5 cm) of soil were sampled to avoid the vertical heterogeneity in microbial communities attributable to soil horizon development, as recommended by Sigler and Zeyer (2002) and Rime et al. (2015). Soil samples were placed in Whirl-Pak® bags and immediately frozen at -20° C for shipment and storage until processing in the laboratory.

DNA extraction

Genomic DNA extraction was performed independently from the 3 different samples in each succession stage (same soil exposure age) using the PowerMax Soil DNA Isolation Kit (MO BIO Laboratories, Inc.) according to standard procedures. DNA concentrations were determined in a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific™). DNA samples from the same succession stage were pooled in equimolar concentrations for further analysis. Pooled DNA samples of each succession stage were then concentrated using a SpeedVac concentrator (Savant Inc.) and purified using the QiaEX II DNA Purification Kit (Qiagen Laboratories INC.) prior to amplification.

Table 1.1. Characteristics of the studied chronosequences in Pia Glacier forefield located at southern slope (SS) of Cordillera Darwin and Parry Glacier forefield at northern slope (NS) of Cordillera Darwin (Tierra del Fuego, Chile).

GLACIER FOREFIELD	SUCCESSION STAGE (SOIL SURFACE AGE)*	ALTITUDE (m.a.s.l.)	AREA DESCRIPTION
SS	SS0 (1 yr)	38 m.a.s.l.	Bare soils
(54° 46' S, 69° 35' W)	SS1 (4 yrs)	34 m.a.s.l.	Scree glacier forefield featuring pioneer lichens (*Placopsis pycnotheca, Sterocaulon* spp.) and mosses (*Ditrichium cylindricarpum*, *Acroschisma wilsonii*)
	SS2 (7 yrs)	31 m.a.s.l.	Glacier forefield sparsely vegetated with pioneer herbs (*Gunnera magellanica*) featuring lichens (*Sterocaulon* ssp.)
	SS3 (10 yrs)	24 m.a.s.l.	Patchy shrubby-vegetated glacier forefield featuring herbs (*Gunnera magellanica*, *Gaultheria mucronata*, *Empetrum rubrum*) and young *Nothofagus* spp.
	SS4 (19 yrs)	15 m.a.s.l.	Herbaceous and shrubby fully vegetated glacier forefield featuring herbs and bushes (*Gaultheria mucronata*, *Empetrum rubrum*, young *Nothofagus antarctica* and *N. betuloides*)
	SS5 (34 yrs)	2 m.a.s.l.	Forest (*Nothofagus antarctica*, *N. betuloides*)
NS	NS0 (5 yrs)	210 m.a.s.l.	Bare soils
(54° 41' S, 69° 23' W)	NS1 (18 yrs)	175 m.a.s.l.	Unvegetated scree glacier forefield featuring pioneer lichens (*Sterocaulon* spp.) and mosses (*Dendroligotrichum squamosum*, *Andreaea laxifolia*)
	NS2 (20 yrs)	152 m.a.s.l.	Glacier forefield sparsely vegetated with pioneer herbs (*Gunnera magellanica*, *Uncinia tenuis*)
	NS3 (26 yrs)	136 m.a.s.l.	Patchy shrubby-vegetated glacier forefield featuring herbs (*Gunnera magellanica*, *Gaultheria mucronata*, *Empetrum rubrum*)
	NS4 (66 yrs)	90 m.a.s.l.	Forefield featuring herbs, shrubs (*Gaultheria mucronata*, *Empetrum rubrum*) and young patchy forest (*Nothofagus antarctica* and *N. betuloides*)

* Obtained from Sancho et al. (2011) and Arróniz-Crespo et al. (2014).

PCR amplification and pyrosequencing

Soil bacterial, fungal and algal communities from the different succession stages were determined by one-way tag-encoded 454 amplicon pyrosequencing. The bacterial *16S* rDNA V1-V3 gene region was amplified using the primer pair 27F (Lane et al. 1985) and 518R (Muyzer et al. 1993). Fungal internal transcribed spacers (ITS) 1 and 2, as well as the *5.8S* ribosomal RNA gene sequence were amplified using the ITS1F and ITS4 primer pair (Gardes and Burns 1993). The algal RuBisCo *rbc*L gene fragment was amplified using the primer pair Rh1 and Rbc590 (Hayden and Waaland 2002). PCR specifications are detailed in the Supporting information 1. Posterior PCRs and 454 pyrosequencing were carried out at the NGS-Genomic Service of the Fundación Parque Científico de Madrid (FPCM) according to their established protocols (detailed in Supporting information 2).

Raw data from sequencing were deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) under accession number SRP052222.

Pyrosequencing data processing

Reads were initially filtered based on a perfect match with MID barcodes and on base quality score ($Q \geq 25$, following Roche standard specifications for 454 GS FLX Titanium). These reads were then processed using a custom BioPython script, which assigns the reads to bacterial, fungal or algal datasets ($\leq$ 2 nucleotides mismatch to primer sequences) and trims tags, adapters and MID barcodes. Reads below a minimum length ($\leq$ 250 bp for bacteria, $\leq$ 400 bp for fungi and $\leq$ 450 bp for algae) and those containing ambiguous nucleotides ('Ns') and/or homopolymers longer than 8 bp were removed from the dataset using the 'trim.seqs' command in MOTHUR v1.22.2 (Schloss et al. 2009). Remaining reads were clustered at 99% of sequence similarity and singletons and chimeric sequences successively removed using USEARCH v6.0.307 (Edgar 2010). Sequence reads were then clustered into operational taxonomic units (OTUs) at the 97% similarity level using the implemented UCLUST algorithm. 'Consensus sequences' generated by the UCLUST algorithm for each OTU were appointed as representative sequences for subsequent analyses. Finally, datasets were independently rarefied to even sequencing depth (885, 1,844 and 5,697 reads for bacteria, fungi and algae per sample, respectively) by random selection (without

replacement), using the 'multiple_rarefactions' command in QIIME (Caporaso et al 2010).

Sequence data

A total of 441,157 reads were obtained for the SS and 439,935 for the NS chronosequences. After removing poor-quality, incorrectly barcoded, short and potentially chimeric sequences, this left 26,517 bacterial sequences, 67,651 fungal sequences and 95,661 algal sequences suitable for analysis. These sequences clustered (97% similarity cut-off) into 3,299 bacterial OTUs, 1,310 fungal OTUs and 240 algal OTUs.

Community structure

Sequencing depth for each type of organism and succession stage in each chronosequence was tested by constructing rarefaction curves using the R package iNEXT for the interpolation and extrapolation of species diversity (Chao et al. 2013; Hsieh et al. 2013). Taxonomic affinities for representative bacterial OTU sequences were assigned using the Ribosomal Database Project's (RDP) Naïve Bayesian rRNA Classifier (Wang et al. 2007) against the RDP database (Maidak et al. 1996). OTU's affinities reported as 'cyanobacteria/chloroplast' were further assigned a taxonomic identity using the 'megablast' algorithm against nr/nt database (NBCI). Representative fungal OTU sequences were assigned a taxonomic identity using the 'BLASTn' algorithm (NCBI) by searching against the UNITE database (Abarenkov et al. 2010). Algal OTU taxon affinities were determined using the 'megablast' algorithm against nr/nt database (NBCI). Matches showing cover > 90% and the highest identity values were chosen after exclusion of unclassified accessions. Higher levels of phylogenetic adscription for each algal OTU were assigned following UniProt Consortium (www.uniprot.org/taxonomy). Bacterial sequences identified as eukaryotic organelle DNA (mitochondria or chloroplast) or as Archaea were removed along with non-fungal or non-algal sequence identities. Simple Correspondence Analyses (CA) were carried out for each type of organism in Stata software v14.0 to test conditional independence relationships between major microbial groups and succession stages.

Microbial diversity

Bacterial, fungal and algal OTU richness (*S*), Shannon diversity index (*H'*) and Pielou's evenness index (*J'*) were calculated per succession stage using the vegan package v2.0-0 in R for community ecology (Oksanen et al. 2013).

Turnover rates for microbial communities in the chronosequences were calculated using the Bray-Curtis dissimilarity index implemented in the vegan package in R. This turnover index ranges from 0 (two sites sharing all OTUs) to 1 (two sites sharing no OTU). Turnover rates per year were calculated dividing Bray-Curtis index values by the difference in estimated soil surface age (years) between consecutive succession stages to examine the speed of OTU turnover along the chronosequence following Wu et al. (2012). These rates were expressed as percentages.

OTU ribbon maps, based on shared OTUs, were built from rarefied datasets to represent connectivity among the soil microbial communities of different succession stages in both glacier forefields. Shared OTUs abundances using rarefied and non-rarefied datasets were not markedly different (data not shown). Data were tabulated using the Reshape2 package v1.4 in R (Wickham 2014) and then represented in the form of circular layouts by CIRCOS software (Krzywinski et al. 2009). In order to check the level of similarity among succession stages (shared OTUs) Multidimensional Scaling (MDS) was performed using Euclidean distances and the PROXSCAL algorithm in SPSS v.23.0.

Previously to β-phylodiversity analysis, bacterial and algal datasets were aligned using MUSCLE v.3.8.31 (Edgar et al. 2004). Maximum likelihood (ML) phylogenetic analyses were performed by means of RAxML v7.2.7 (Stamatakis 2006) using the GAMMACAT substitution model, implemented in the CIPRES Science Gateway (Miller et al. 2010). Fungal dataset was discarded in β-phylodiversity analyses since the typical high number of polymorphisms in ITS sequences prevents the build of reliable alignments. Using ML tree hypotheses, phylogenetic β-diversity was calculated and presented as phylogenetic lineages in a phylogenetic distance matrix constructed using a weighted normalized UniFrac metric measure (Lozupone and Knight 2005) using the Fast UniFrac suite (Hamady et al. 2010). To detect broad trends in similarities and differences in microbial lineages according to succession stages, a principal coordinates analyses (PCoA) was performed based on the UniFrac metric measure for both bacterial and algal datasets (Lozupone and Knight 2005).

1.3 Results

Bacterial community structure

Bacterial communities were dominated by *Proteobacteria* (52.7% OTUs, 43.3% sequences), with *Alphaproteobacteria* emerging as the most represented class. *Acidobacteria* (16.7% OTUs, 31.9% sequences). The *Actinobacteria* (7.3% OTUs, 5.5% sequences), and *Sphingobacteria* (7% OTUs, 3.8% sequences), also showed remarkable abundance and OTU richness along the chronosequences.

The same dominant bacterial classes and orders (the latter hereafter given between brackets) were represented on both chronosequences. However, differences in bacterial community composition according to distance from the glacier terminus (Table 1.2), as well as statistically supported associations of specific bacterial groups with given succession stages (Fig. 1.1, CA with variance explained of 89.02%, $p \leq 0.001$), were detected. *Alphaproteobacteria* (*Rhizobiales*) showed increasing OTU richness and abundance with distance from the glacier terminus (Table 1.2) and association to SS3 and SS4 succession stages (Fig. 1.1). In contrast, *Cyanobacteria* (*Oscillatoriales* and *Nostocales*) showed a higher abundance close to the glacier terminus (SS0, NS0) and closer association to SS0 (Fig. 1.1), while they were nearly absent at later succession stages (Table 1.2). *Actinobacteria* (*Acidimicrobiales* and *Solirubobacteriales*) also showed the highest OTU richness and abundance values at SS0 and NS0 and a markedly fell of richness and abundance with increasing distance fromthe glacier terminus in the NS chronosequence (Table 1.2). These results were supported by their location in the CA ordination (Fig. 1.1). Contrarily, OTU richness and abundance values for *Acidobacteria* (*Acidobacteriales*) were markedly lower at SS0 and NS0 than at remaining succession stages (Table 1.2, Fig. 1.1). *Candidatus Saccharibacteria* was associated to SS0, *Opitutae* to SS1 and SS2 and *Acidobacteria* to SS5 (Fig. 1.1).

Table 1.2. Bacterial community composition along the southern slope (SS) and northern slope (NS) chronosequences

Succession stage	*α-Proteobacteria*		*β-Proteobacteria*		*γ-Proteobacteria*		*δ-Proteobacteria*		*Cyanobacteria*		*Acidobacteria*		*Sphingobacteria*		*Actinobacteria*		*Opitutae*		*Candidatus Saccharibacteria*		Others	
	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs
SS0	17.5	7.3	4.5	1.1	4	1.1	2.8	0.9	13	41.3	4	3.6	7.9	3.3	20.3	18.7	1.1	0.2	9.6	12.3	15.3	10
SS1	32.8	32.9	11.1	9.8	10.6	17.9	6.2	3.9	0	0	11.4	13.6	9.1	7.2	3.5	2.2	4.7	5.1	0.9	0.6	9.7	6.8
SS2	28.7	29.6	12.5	8.3	7.2	13.4	2.3	1.5	0.4	0.3	15.1	13.1	5.7	7.8	9.8	10.7	3.4	2.7	0.8	0.6	14.3	12
SS3	37.4	40.3	5.3	3.3	7.8	6.3	4	2.2	0	0	18.2	27.8	7.8	5.8	5.1	3.9	2.7	1.7	0.8	0.7	11	8.1
SS4	41.9	46.7	4	1.9	7.9	7.9	2.3	1.1	0	0	20.8	27.3	2.3	1.8	6.6	4.7	2	0.9	1.7	1	10.6	6.6
SS5	33.9	19.9	4.7	1.2	7.8	9.8	1	0.5	0	0	37.5	62.9	2.1	0.7	4.7	1.5	4.2	2	1	0.2	3.1	1.2
NS0	21.2	22.4	7.5	5.9	6.2	4.2	5.1	3.7	10.3	17	6.5	4.8	4.5	4.6	18.5	19.5	1.4	0.9	2.1	2.1	16.8	15
NS1	32.6	31.6	3.6	2.6	8.1	7.7	2.3	1.4	0.5	0.2	28.1	36.7	2.3	1.5	5.4	5.3	2.7	1.8	0.5	0.2	14	10.8
NS2	32.9	33	5.6	3.2	8.2	7.4	3.8	2.2	0	0	24.5	37.3	4.7	2.6	4.1	1.7	1.3	0.8	0.9	0.9	14.1	10.9
NS3	52.8	56.8	3	1.1	5.6	2.9	2.1	0.7	0	0	25.8	34.6	2.6	1.1	2.1	0.8	0	0	0	0	6	1.9
NS4	38.9	43.3	3.7	2.4	6.9	5.6	4.5	2.1	0	0	28	36	2.1	1.2	2.9	1.7	1.9	1.1	1.1	0.5	9.9	6

OTU richness (OTUs) and relative sequence abundance (Seqs) are expressed as a percentage of the total number of OTUs and sequence reads detected for each succession stage (left column), respectively, resolvable to at least the class level (top row). Taxa accounting for <0.5% of relative abundance in >50% of the succession stages and OTUs only resolved at the phylum level are shown in the column designated Others. The number of reads for each succession stage was standardized to a minimum sampling read depth by rarefying through random selection without replacement (885 reads).

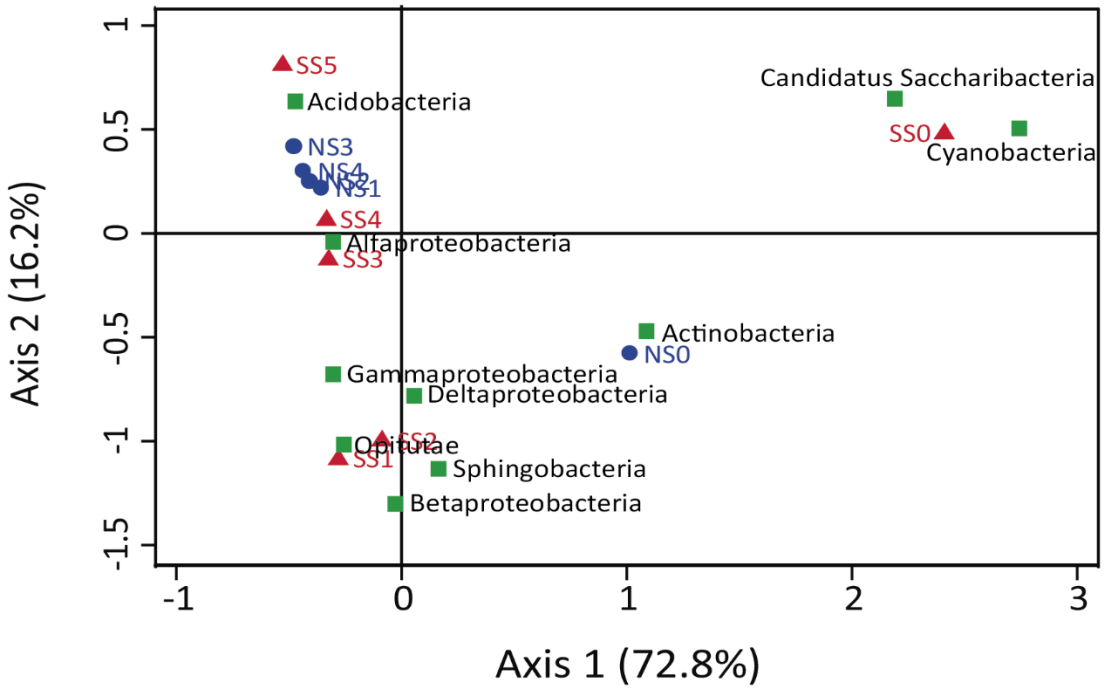


Fig. 1.1. Single correspondence analysis (CA) of the bacterial classes from the different succession stages. Variables explained 89.02% of the variance of the dataset ($p \leq 0.001$). Southern slope succession stages (SS0-SS5) are indicated with red triangles and northern slope succession stages (NS0-NS4) with blue dots. Bacterial classes are indicated by green squares.

Fungal community structure

The phylum *Ascomycota* strongly dominated fungal communities (59.3% OTUs, 55.3% sequences), followed by *Basidiomycota* (23.6% OTUs, 29.9% sequences). Within *Ascomycota*, *Leotiomycetes* was the most abundant class (27.9% OTUs, 18.7% sequences). *Agaricomycetes* (20.3% OTUs, 27.7% sequences) was the most abundant class among the *Basidiomycota*.

Fungal community structure varied across the SS and NS chronosequences (Table 1.3), with changes in composition statistically supported (Fig. 1.2, CA with a variance explained of 57.07%, $p \leq 0.001$). Clear trends according to distance from the glacier terminus were not observed in abundance values for the most represented classes, *Leotiomycetes* (*Helotiales*) and *Agaricomycetes* (*Cantharellales*, *Agaricales* and *Sebacinales*). However, OTU richness and abundance values recorded for other classes such as *Lecanoromycetes* (*Agyriales*) and *Tremellomycetes* (*Trichosporonales* and

Tremellales) decreased with distance from the glacier terminus (Table 1.3) and were associated in CA (Fig. 1.2) with lower soil surface ages (NS0, SS1 and SS2). Similarly, the class *Chytridiomycetes* (*Rhizophydiales* and *Rhizophylictidiales*) was almost exclusively detected at sites NS0, SS0 and SS1 (Table 1.3) and associated to SS0 (Fig. 1.2). On the other hand, *Archaeorhizomycetes* (*Archaeorrhizomycetales*) was the dominant class at NS3 and NS4 (supported by CA analysis, Fig. 1.2) but virtually absent from the remaining succession stages.

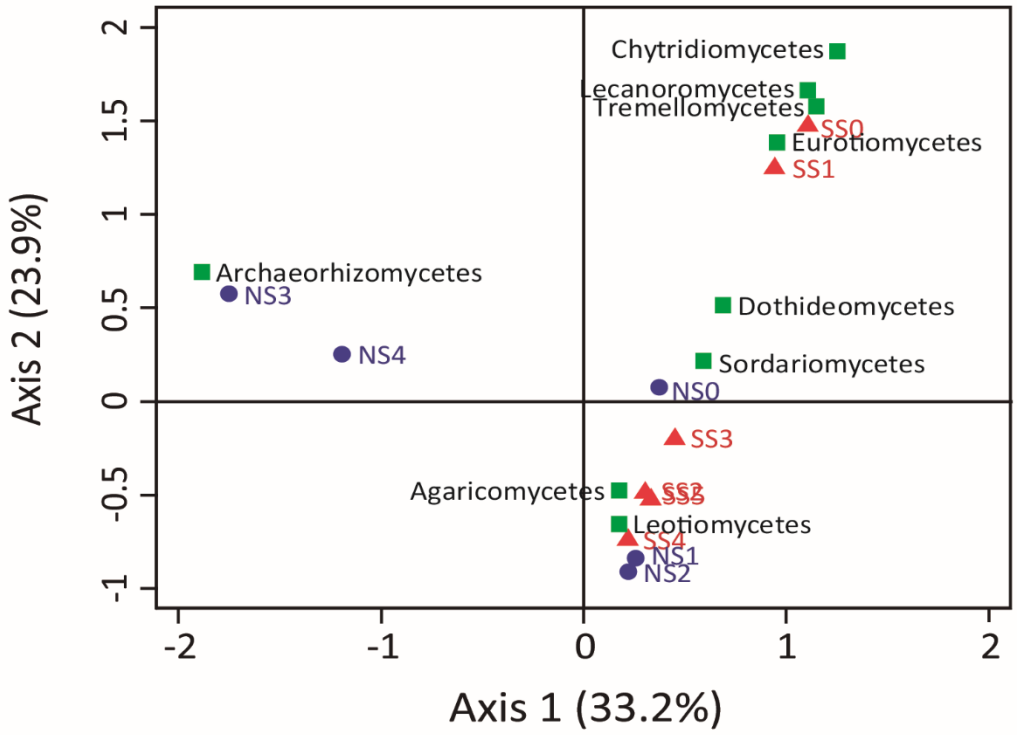


Fig. 1.2. Single correspondence analysis (CA) of the fungal classes from the different succession stages. Variables explained 57.07% of the variance of the dataset ($p \leq 0.001$). Southern slope succession stages (SS0-SS5) are indicated with red triangles and northern slope succession stages (NS0-NS4) with blue dots. Fungal classes are indicated by green squares.

Table 1.3. Fungal community composition along the southern slope (SS) and northern slope (NS) chronosequences

Succession stage	*Chytridiomycetes*		*Dothideomycetes*		*Eurotiomycetes*		*Lecanoromycetes*		*Leotiomycetes*		*Sordariomycetes*		*Archaeorhizomycetes*		*Tremellomycetes*		*Agaricomycetes*		Others	
	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs
SS0	7.6	18	6.7	5.2	3.8	1.9	11.4	9.6	16.2	16.2	3.8	1.1	1	0.2	4.8	15	13.3	2.9	31.4	29.8
SS1	4.7	2.1	0	0	11.3	23.5	13.2	24.5	18.9	5.9	13.2	8.2	0	0	0.9	0.1	12.3	17.3	25.5	18.3
SS2	0	0	5.6	1.4	5.6	0.6	7.4	1.3	13.9	3.2	1.9	0.2	0.9	0.1	0.9	1.1	30.6	37	33.3	55.1
SS3	0.8	0.2	6.2	1.5	3.8	0.5	0	0	14.6	5.5	6.2	2.7	0.8	0.1	5.4	1.6	13.1	8.5	49.2	79.5
SS4	0	0	1.2	0.1	1.2	0.2	0	0	29.3	14.5	8.5	1.8	0	0	0	0	20.7	73.8	39	9.7
SS5	0	0	2.2	0.4	4.4	0.3	5.6	1.3	20	11.5	6.7	11.3	1.1	0.3	2.2	1.3	37.8	57.7	20	16
NS0	12.7	3.4	7.8	12.1	2	0.8	4.9	2.4	16.7	9.3	2	0.2	6.9	3.3	1	0.3	13.7	25.7	32.4	42.6
NS1	0	0	1.8	0.2	10.1	2.9	2.8	0.4	34.9	73	2.8	0.8	0.9	0.1	0.9	0.1	20.2	11.9	25.7	10.6
NS2	0	0	0	0	0.7	0.2	0.7	0.1	61.9	74.9	3.6	0.6	0	0	0.7	0.1	23.7	22.1	8.6	2.1
NS3	0	0	0.9	0.1	10.4	0.8	0.9	0.2	37.7	11.8	2.8	0.7	17.9	74	2.8	0.2	21.7	11.7	4.7	0.5
NS4	0	0	1.5	0.1	2.2	0.9	2.2	1	30.9	17.5	2.9	0.5	22.8	52.9	0.7	0.1	27.2	25.5	9.6	1.4

OTU richness (OTUs) and relative sequence abundance (Seqs) are expressed as a percentage of the total number of OTUs and sequence reads detected for each succession stage (left column), respectively, resolvable to at least the class level (top row). Taxa that account for <0.5% of relative abundance in >50% of the succession stages and OTUs only resolved at the phylum level are shown in the column designated Others. The number of reads for each succession stage was standardized to a minimum sampling read depth by rarefying through random selection without replacement (1844 reads).

Algal community structure

Algal communities were dominated by the class *Trebouxiophyceae* (65% OTUs, 65% sequences) followed by *Chlorophyceae* (20% OTUs, 30.7% sequences). *Trebouxiophyceae* reads were mainly assigned to taxonomical identities within the order *Microthamniales* (18.7% OTUs, 19.5% sequences) and *Chlorophyceae* to the order *Chlamydomonadales* (2.9% OTUs, 24.2% sequences).

Most represented algal orders and genera (the latter hereafter given between parentheses), except the orders *Tetrasporales* and *Desmidiales*, were found at every sampling site in both forefields (Table 1.4), findings corroborated by the spread ordination shown by CA (Fig. 1.3, variance explained of 76.38%, $p \leq 0.001$). The orders *Microthamniales* (*Stichococcus* and *Trebouxia*) and *Chlorelalles* (*Chorella*, *Chloroidium* and *Oocystis*) showed similar OTU richness and abundances along the chronosequences. However, OTUs assigned to the order *Prasiolales* (*Prasiola*) showed greater abundance at SS0, NS0 and SS3 (Table 1.4) and a main association with them in CA (Fig. 1.3). *Prasiolales* OTUs identified in the youngest succession stages of each forefield (SS0, NS0) were mainly ascribed to *Prasiola calophylla*, while in SS3 were assigned to different *Prasiola* species (Table 1.4). The order *Chlamydomonadales* (*Eudorina*) featured lower OTU richness and abundance values in initial succession stages compared to older stages. *Desmidiales* (*Actinotaenium*) were associated with NS4 and both segregated from the rest in CA (Fig. 1.3), coinciding with the highest values of abundance and richness of the order at this site (Table 1.4).

Table 1.4. Algal community composition along the southern slope (SS) and northern slope (NS) chronosequences

Succession stage	*Chaetophorales*		*Chlamydomonadales*		*Sphaeropleales*		*Tetrasporales*		*Chlorellales*		*Microthamniales*		*Prasiolales*		*Desmidiales*		Others	
	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs	OTUs	Seqs
SS0	4.2	0.8	2.1	0.6	4.2	1.7	4.2	0.6	29.2	9.2	18.8	3.9	12.5	79	0	0	25	4.2
SS1	7.8	9.6	4.4	41.6	6.7	3	3.3	2.2	25.6	14.2	20	16.2	1.1	0.3	1.1	0.8	30	12.2
SS2	4	0.4	4	22.5	10	1.3	2	0.2	18	2.6	18	70.8	4.0	0.2	0	0	40	2.1
SS3	9.6	3.2	3.8	7.5	1.9	0.1	0	0	17.3	5.9	21.2	14.8	5.8	30.5	1.9	1.5	38.5	36.7
SS4	13.3	3.8	6.7	23.1	4.4	0.1	0	0	20	8.8	13.3	31.9	6.7	10.6	0	0	35.6	21.7
SS5	2	0.1	4.1	44.9	2	0.1	0	0	24.5	5.8	22.4	5.8	4.1	1.1	0	0	40.8	42.2
NS0	4.4	0.8	4.4	2.4	6.7	11.1	0	0	24.4	12.5	23.3	12.4	3.3	25.2	2.2	0.2	31.1	35.4
NS1	4.1	0.1	4.1	18.6	2.7	0.2	1.4	0.1	20.5	9.7	16.4	19.5	5.5	0.4	1.4	0.2	43.8	51.2
NS2	4.3	1.3	2.9	37	4.3	1.5	1.4	0.2	18.8	5.8	17.4	11.2	1.4	0.2	2.9	1.7	46.4	41.1
NS3	10.1	3.8	2.5	19.1	3.8	2.1	0	0	16.5	4.6	17.7	12.5	2.5	0.5	5.1	1.7	41.8	55.6
NS4	12.5	9.1	6.3	37.8	3.1	0.6	3.1	0.2	21.9	9.2	18.8	7.9	6.3	0.1	9.4	29.3	18.8	5.8

OTU richness (OTUs) and relative sequence abundance (Seqs) are expressed as a percentage of the total number of OTUs and sequence reads detected for each succession stage (left column), respectively, resolvable to at least the order level (top row). Taxa that account for <0.5% of relative abundance in >50% of the succession stages (along the chronosequence) and OTUs only resolved at the sub-phylum level are shown in the column designated 'Others'. The number of reads for each succession stage was standardized to minimum sampling read depth by rarefying through random selection without replacement (5697 reads).

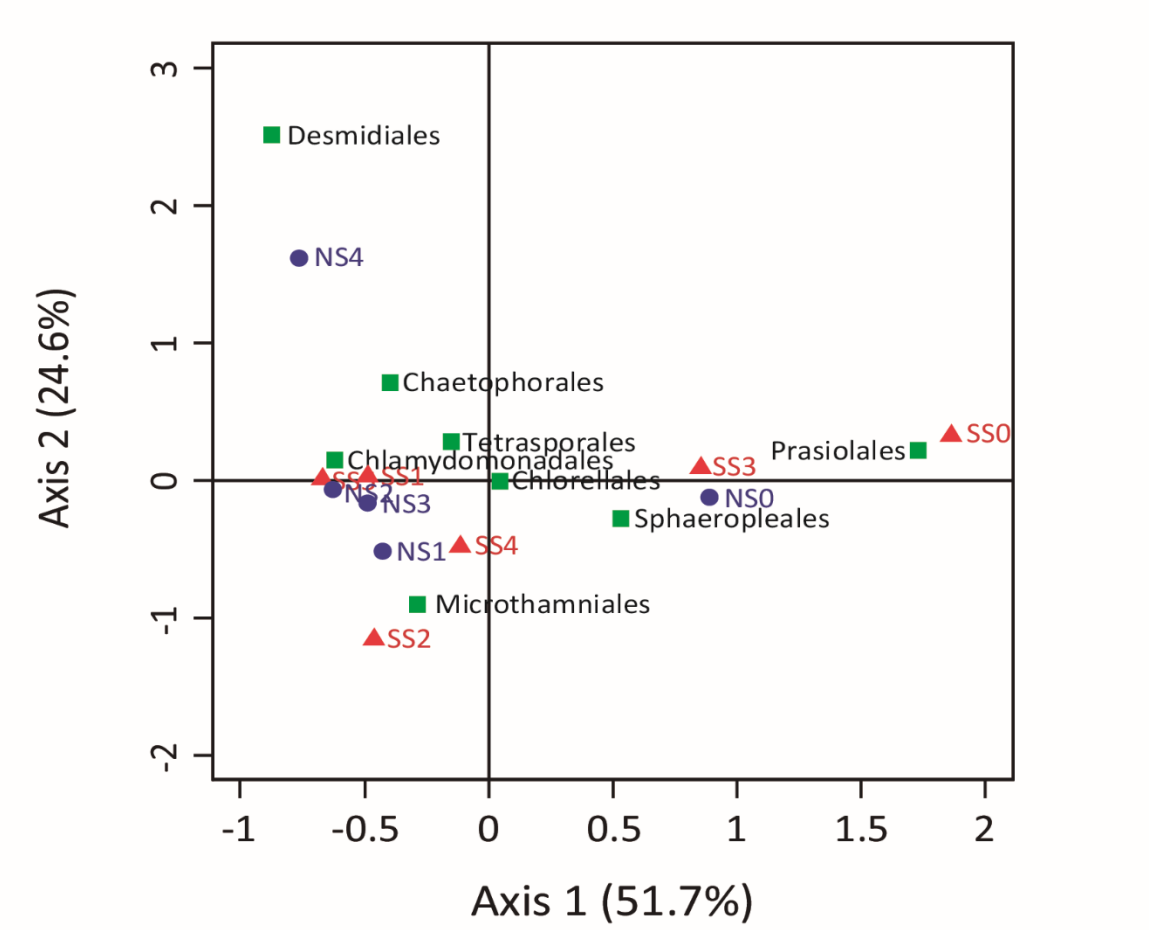


Fig. 1.3. Single correspondence analysis (CA) of the algal orders from the different succession stages. Variables explained 76.38% of the variance of the dataset ($p \leq 0.001$). Southern slope succession stages (SS0-SS5) are indicated with red triangles and northern slope succession stages (NS0-NS4) with blue dots. Algal orders are indicated by green squares

Microbial richness and α-diversity

Our rarefaction curves indicated a satisfactory sequencing depth for algae at all sites and for bacteria and fungi at most (Supporting information Fig. 1.1). Richness (*S*), Shannon diversity (*H'*) and Pielou's evenness (*J'*) estimators calculated for bacterial, fungal and algal communities did not show clear successional trends along both chronosequences (Table 1.5). For bacterial communities, the lowest values of *S*, *H'* and *J'* were recorded for the SS chronosequence at SS0 and SS5 and for the NS at NS3. Fungal and algal communities showed lower *S*, *H'* and *J'* values than bacterial ones across both

forefields. The lowest fungal *S*, *H'* and *J'* values were found at SS4 and NS3, while for algal communities, the lowest values were recorded at SS2 for SS chronosequence and NS4 for NS.

Table 1.5. Estimated OTU richness (*S*), diversity (*H'*) and evenness (*J'*) indices for bacterial, fungal and algal communities detected along the southern slope (SS) and northern slope (NS) chronosequences.

Succession stage	Bacteria			Fungi			Algae		
	S	*H'*	*J'*	*S*	*H'*	*J'*	*S*	*H'*	*J'*
SS0	182	3.5	0.67	106	3.8	0.81	48	1.77	0.46
SS1	344	5.2	0.89	107	3.08	0.66	93	2.7	0.6
SS2	267	5.18	0.93	111	2.23	0.47	51	1.2	0.3
SS3	375	5.35	0.9	135	3.48	0.71	54	2.47	0.62
SS4	303	5.02	0.88	83	2.02	0.46	50	2.36	0.6
SS5	192	3.94	0.75	93	2.23	0.49	55	2.01	0.5
NS0	297	5.34	0.94	102	3.13	0.68	90	2.67	0.59
NS1	223	5.01	0.93	109	2.16	0.46	76	2.4	0.55
NS2	320	5.02	0.87	140	2.43	0.49	70	2.38	0.56
NS3	233	4.09	0.75	106	1.94	0.42	80	2.73	0.62
NS4	375	5.22	0.88	138	3.17	0.64	32	1.91	0.55

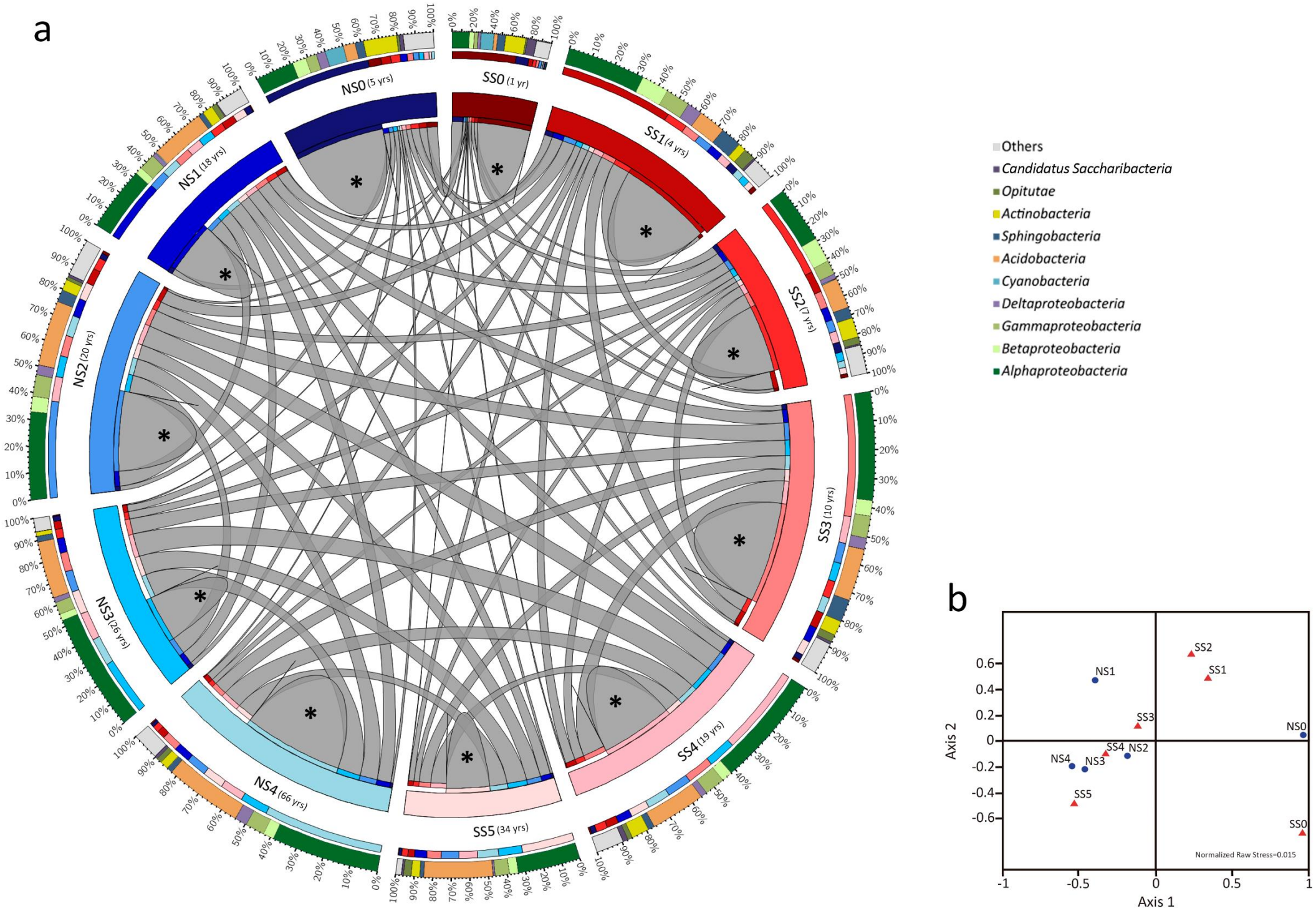
Bacterial OTU turnover rates and distributions

For the SS glacier forefield, highest Bray-Curtis dissimilarity indices (BC_{ij}) and most rapid turnover rates per year (32.3%) were detected between SS0 and SS1 (Table 1.6), while the slowest turnover rate per year was found between SS4 and SS5 (5.4%). Similarly, along the northern slope chronosequence, the highest BC_{ij} was obtained between NS0 and NS1. However, the highest turnover rate per year (35.4%) was found between NS1 and NS2 (Table 1.6). In contrast, the slowest turnover rate per year (1.7%) was observed for NS3 versus NS4 (Table 1.6). In both forefields, BC_{ij} decreased along the chronosequence before reaching the full forest stage. However, the turnover rate per year did not follow this pattern.

Table 1.6. Bray-Curtis dissimilarity indices (BC_{ij}) and turnover rates per year (expressed as percentage) between consecutive succession stages of bacterial, fungal and algal communities along the southern slope (SS) and northern slope (NS) chronosequences.

Succession stages	BC_{ij}			Turnover rate per year		
	Bacteria	Fungi	Algae	Bacteria	Fungi	Algae
SS0-SS1	0.97	0.96	0.91	32.3	32	30.4
SS1-SS2	0.77	0.84	0.65	25.5	28.1	21.7
SS2-SS3	0.85	0.97	0.81	28.3	32	26.9
SS3-SS4	0.72	0.95	0.47	8	10.5	5.2
SS4-SS5	0.81	0.97	0.55	5.4	6.5	3.7
NS0-NS1	0.93	0.99	0.85	7.1	7.6	6.5
NS1-NS2	0.71	0.91	0.46	35.4	45.5	23.2
NS2-NS3	0.75	0.89	0.32	12.5	14.8	5.3
NS3-NS4	0.68	0.89	0.70	1.7	2.2	1.7

The bacterial OTU ribbon map (Fig. 1.4a) illustrates the high abundance of bacterial shared OTUs between different succession stages (ribbons). The high abundance of unique OTUs at every succession stage is also revealed (42.8±10.7%, asterisks in Fig. 1.4a). Succession stages closest to the glacier terminus, SS0 and NS0, shared more OTUs (mainly assigned to *Alphaproteobacteria*, *Cyanobacteria* and *Actinobacteria*) between them, than with any other site. The highest abundance of unique OTUs (68% and 62% respectively, Fig. 1.4a) were also found in NS0 and SS0. The differentiation of SS0 and NS0 was corroborated by their segregation in multidimensional scaling (MDS) ordination (Fig. 1.4b). It is also remarkable the low number of shared OTUs between SS0 and NS0 and the subsequent succession stage in their own chronosequence (pairs SS0-SS1 and NS0-NS1; ribbons in Fig. 1.4a), and their distant location in MDS ordination (Fig. 1.4b). The subsequent succession stages in the southern chronosequence, SS1 and SS2, showed a higher abundance of shared OTUs between them than with any other site (ribbons and inner bar-plots of external rings in Fig. 1.4a), which coincided with the observed segregation of them from the rest of the succession stages in MDS (Fig. 1.4b). A close location of the rest of the succession stages (SS3, SS4, SS5, NS2, NS3 and NS4) in MDS ordination (Fig. 1.4b) and similar abundances of shared OTUs between them (ribbons and Supporting information Table 1.1) were also observed.


a
NS0 (5 yrs)
SS0 (1 yr)
SS1 (4 yrs)
NS1 (18 yrs)
SS2 (7 yrs)
NS2 (20 yrs)
SS3 (10 yrs)
NS3 (26 yrs)
SS4 (19 yrs)
NS4 (66 yrs)
SS5 (34 yrs)
Others
Candidatus Saccharibacteria
Opitutae
Actinobacteria
Sphingobacteria
Acidobacteria
Cyanobacteria
Deltaproteobacteria
Gammaproteobacteria
Betaproteobacteria
Alphaproteobacteria
b
SS2
SS1
NS1
SS3
NS0
SS4
NS2
NS4
NS3
SS5
SS0
Normalized Raw Stress=0.015
Axis 2
Axis 1

◀ **Fig. 1.4. (a)** Ribbon map of bacterial OTUs detected along the southern slope (SS0-SS5, red colour-scheme) and northern slope (NS0-NS4, blue colour-scheme) glacier forefield chronosequences. In the centre of the figure, grey ribbons represent the number of OTUs shared between two succession stages (indicated by the corresponding colours at the ribbon ends). Numbers of unique OTUs are indicated by individual ribbons (asterisks). The length of the coloured ring segments represents the number of OTUs in each succession stage. These segments are ordered clockwise (SS, right half of the figure) and counterclockwise (NS, left half of the figure), from younger to older soil surface ages. The two coloured bar-plot rings situated externally show the percentages of OTUs from the total number of OTUs of each succession stage of unique and shared OTUs between succession stages (inner ring) and OTUs ascribed to different bacterial classes (outer ring, legend). **(b)** Multidimensional scaling ordination plot derived from a similarity matrix between succession stages based on bacterial OTUs community composition along southern and northern slopes glacier forefield chronosequences. Southern slope succession stages (SS0-SS5) are indicated with red triangles and northern slope succession stages (NS0-NS4) with blue dots.

Fungal OTU turnover rates and distributions

The turnover rate (BC_{ij}) was high (0.99 to 0.89) and similar across the entire chronosequence (Table 1.6). As for the bacteria, turnover rates per year did not follow the same pattern in both glacier forefields. In the SS chronosequence, the most rapid turnover rates per year were detected between the first three succession stages while in NS most rapid rates were noted for NS0 versus NS1 (Table 1.6).

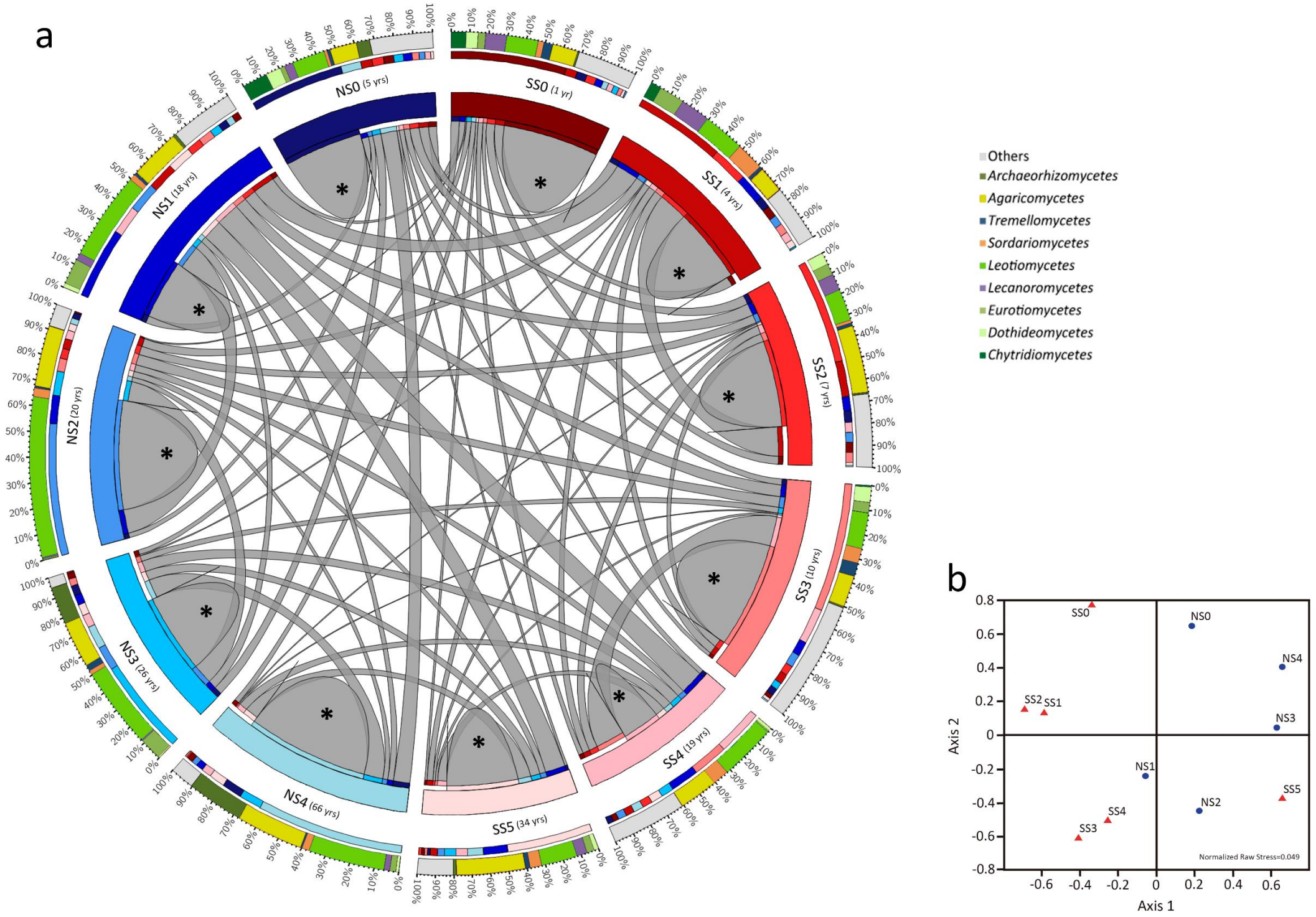
The fungal OTU ribbon map (Fig. 1.5a) indicates that all the succession stages from both glacier forefields shared fungal OTUs, except pairs SS1-NS4 and SS2-NS3 which did not show any shared OTU (ribbons). Ribbon map also depicts the remarkable abundance of unique OTUs found at each succession stage (48$\pm$12%, asterisks in Fig. 1.5a), coinciding with a spread location of the stages over the MDS ordination space (Fig. 1.5b). The succession stages closest to the glacier terminus, SS0 and NS0, were segregated from the rest in the MDS ordination (Fig. 1.5b), as in bacterial analysis. In fact, SS0 and NS0 were distantly located from the immediately subsequent succession stages, SS1 and NS1, respectively (Fig. 1.5b). However, the highest proportions of unique OTUs appeared at SS0 (64%, most of them belonging to *Chytridiomycetes*) and NS4 (62%,

most of them belonging to *Archaeorhizomycetes*). SS1 and SS2 shared a higher number of fungal OTUs between them than with any other succession stage (ribbons and inner bar-plots of external rings in Fig. 1.5a), supported by their nearby location in the MDS ordination (Fig. 1.5b), analogously to bacterial analysis. Higher abundances of fungal shared OTUs were found comparing succession stages from the same glacier than between sites from different glacier (ribbons and Supporting information Table 1.1), corroborated by the observed trend of segregation along Axis 1 in MDS ordination of succession stages from both glaciers (Fig. 1.5b).

Algal OTU turnover rates and distributions

Significantly lower BC_{ij} values were noted for algal communities than for the bacterial and fungal communities (Table 1.6). However, clear trends in BC_{ij} were not detected for each glacier's chronosequence. The two chronosequences showed different turnover patterns in terms of annual rates. The most rapid changes in turnover rate per year (>20%) were detected among the first three succession stages at SS, but only between NS1 and NS2 at NS (Table 1.6).

The algal OTU ribbon map (Fig. 1.6a) shows the high abundance of shared algal OTUs found comparing different succession stages (>94% on average, ribbons and inner bar-plots of external rings) and the low abundance of unique OTUs (asterisks in Fig. 1.6a), which ranged from 1% (SS3, SS4, NS2 and NS4) to 6% (SS1 and NS0). These abundance patterns differed to these found for bacterial and fungal communities. All sites shared high number of OTUs with sites from both glacier forefields (ribbons and inner bar-plots of external rings in Fig. 1.6a, Supporting information Table 1.1) and showed similar abundance of shared OTUs. The algal OTU's ubiquity was also supported by the central position and proximity of the most of the succession stages in the MDS ordination (Fig. 1.6b). The most distant sites in MDS ordination (Fig. 1.6b) were SS0, featured by a high abundance of unique OTUs belonging to *Prasiolales* (outer bar-plots of external rings in Fig. 1.6a), and NS4, showing a high abundance of unique OTUs belonging to *Desmidiales* (outer bar-plots of external rings in Fig. 1.6a).

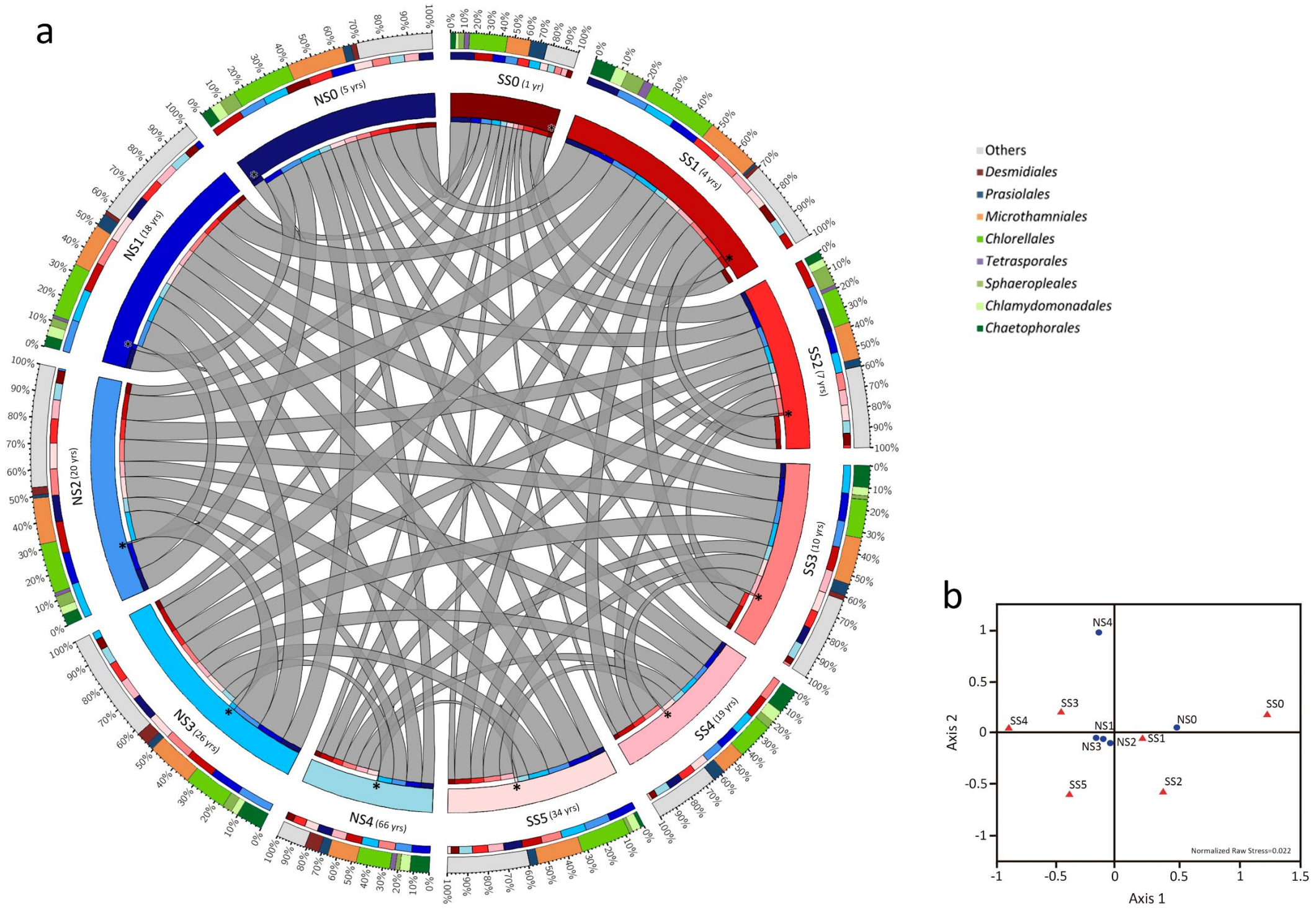

a
NS0 (5 yrs)
SS0 (1 yr)
NS1 (18 yrs)
SS1 (4 yrs)
NS2 (20 yrs)
SS2 (7 yrs)
NS3 (26 yrs)
SS3 (10 yrs)
NS4 (66 yrs)
SS4 (19 yrs)
SS5 (34 yrs)
Others
Archaeorhizomycetes
Agaricomycetes
Tremellomycetes
Sordariomycetes
Leotiomycetes
Lecanoromycetes
Eurotiomycetes
Dothideomycetes
Chytridiomycetes
b
SS0
NS0
NS4
SS2
SS1
NS3
NS1
SS5
NS2
SS4
SS3
Normalized Raw Stress=0.049
Axis 1
Axis 2

◄ **Fig. 1.5. (a)** Ribbon map of fungal OTUs detected along the southern slope (SS0-SS5, red colour-scheme) and northern slope (NS0-NS4, blue colour-scheme) glacier forefield chronosequences. In the centre of the figure, grey ribbons represent the number of OTUs shared between two succession stages (indicated by the corresponding colours at the ribbon ends). Numbers of unique OTUs are indicated by individual ribbons (asterisks). The length of the coloured ring segments represents the number of OTUs in each succession stage. These segments are ordered clockwise (SS, right half of the figure) and counterclockwise (NS, left half of the figure), from younger to older soil surface ages. The two coloured bar-plot rings situated externally show the percentages of OTUs from the total number of OTUs of each succession stage of unique and shared OTUs between succession stages (inner ring) and OTUs ascribed to different fungal classes (outer ring, legend). **(b)** Multidimensional scaling ordination plot derived from a similarity matrix between succession stages based on fungal OTUs community composition along southern and northern slopes glacier forefield chronosequences. Southern slope succession stages (SS0-SS5) are indicated with red triangles and northern slope succession stages (NS0-NS4) with blue dots.

Phylogenetic ordination of communities

Bacterial PCoA of the weighted UniFrac distance matrix (Fig. 1.7a), representing the highest percentage of the variance found in this dataset (62.21%; axis P1=41.71%, axis P2=21.5%), segregated the youngest succession stages in each forefield (SS0 and NS0) from the rest ones. Moreover, axis P2 divided the SS succession stages from those of the NS, with the exception of SS5.

Algal PCoA (Fig. 1.7b) represented 82.83% of the total variance found in the data (axis P1=52.22%, axis P2=30.61%). Similarly to bacterial PCoA, the youngest succession stages, SS0 and NS0, clustered together and segregated from the rest. Among the remaining succession stages, those in the same forefield clustered nearby, with the exceptions of SS5, clustering with the NS sites, and NS4, appearing separated from the rest.


a
NS0 (5 yrs)
SS0 (1 yr)
SS1 (4 yrs)
NS1 (18 yrs)
SS2 (7 yrs)
NS2 (20 yrs)
SS3 (10 yrs)
NS3 (26 yrs)
SS4 (19 yrs)
NS4 (66 yrs)
SS5 (34 yrs)
Others
Desmidiales
Prasiolales
Microthamniales
Chlorellales
Tetrasporales
Sphaeropleales
Chlamydomonadales
Chaetophorales
b
NS4
SS3
SS0
SS4
NS1
NS0
NS3
NS2
SS1
SS5
SS2
Normalized Raw Stress=0.022
Axis 1
Axis 2

◀ **Fig. 1.6. (a)** Ribbon map of algal OTUs detected along the southern slope (SS0-SS5, red colour-scheme) and northern slope (NS0-NS4, blue colour-scheme) glacier forefield chronosequences. In the centre of the figure, grey ribbons represent the number of OTUs shared between two succession stages (indicated by the corresponding colours at the ribbon ends). Numbers of unique OTUs are indicated by individual ribbons (asterisks beneath). The length of the coloured ring segments represents the number of OTUs in each succession stage. These segments are ordered clockwise (SS, right half of the figure) and counterclockwise (NS, left half of the figure), from younger to older soil surface ages. The two coloured bar-plot rings situated externally show the percentages of OTUs from the total number of OTUs of each succession stage of unique and shared OTUs between succession stages (inner ring) and OTUs ascribed to different algal orders (outer ring, legend). **(b)** Multidimensional scaling ordination plot derived from a similarity matrix between succession stages based on algal OTUs community composition along southern and northern slopes glacier forefield chronosequences. Southern slope succession stages (SS0-SS5) are indicated with red triangles and northern slope succession stages (NS0-NS4) with blue dots.

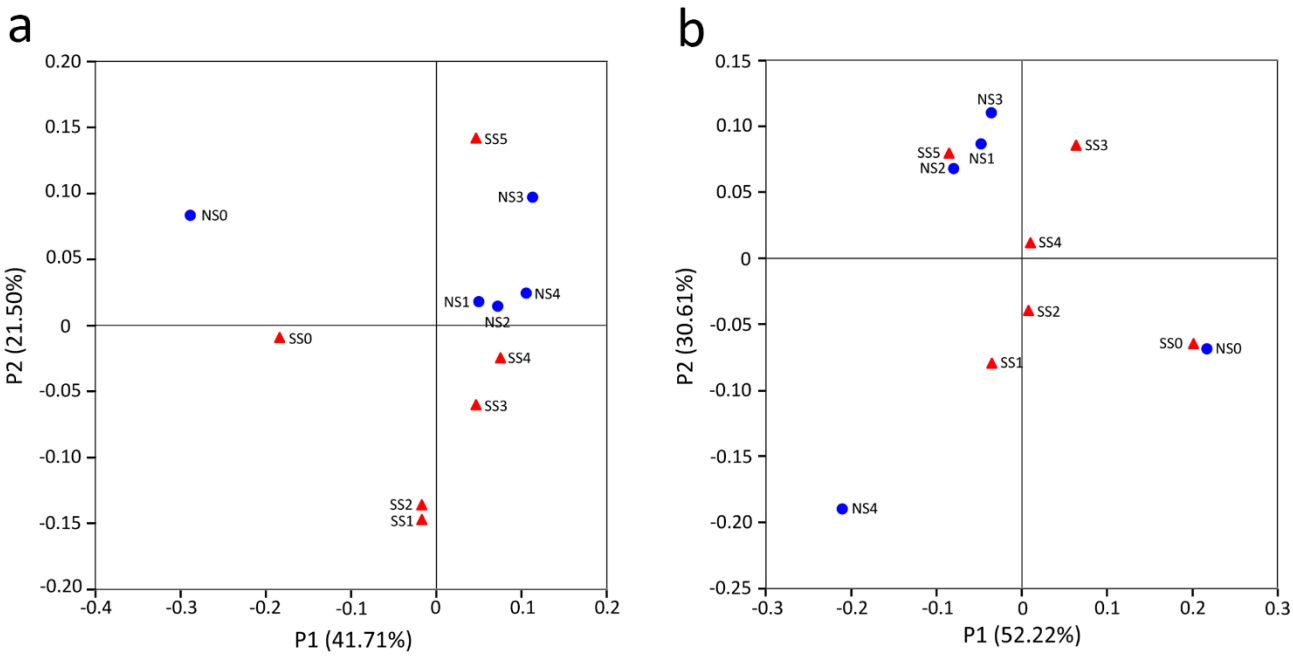


Fig. 1.7. Principal coordinates analysis (PCoA) based on weighted and normalized UniFrac metric measures for succession stages along both forefield chronosequences. Southern slope succession stages (SS0-SS5) are indicated with red triangles and northern slope succession stages (NS0-NS4) with blue dots. (a) Bacterial communities; axes P1 and P2 represent 62.21% of the total variance in the data. (b) Algal communities; axes P1 and P2 represent 82.83% of the total variance in the data. Individual variance represented by each axis appears between parentheses in (a) and (b) (note the differences in scales).

1.4 Discussion

This study examined the structure and succession dynamics of bacterial, fungal and algal communities in soil chronosequences generated by the retreat of two glaciers located on slopes with opposing aspects on the Cordillera Darwin. The study revealed that exposure to different moisture regimes, as a result of aspect, was associated to soil colonization of the forefields from the two slopes showing not only different plant succession rates, but also displaying different microbial temporal dynamics. However, it was also shown that soil chronosequences on both slopes also shared succession patterns for each one of the three taxonomic groups examined. Hence, some taxa were widely distributed across the chronosequences while others were specific to given succession stages and not to the soil surface ages. To the best of our knowledge, this is the first study to simultaneously address bacterial, fungal and algal diversity and composition in different glacier forefield soil chronosequences within potentially reach of the same regional pool of microorganisms.

The bacterial succession patterns detected were analogous to those described for glacier forefields worldwide, with *Proteobacteria* as the dominant bacterial phylum (Nemergut et al. 2007; Zumsteg et al. 2012; Brown and Jumpponen 2014). This widespread distribution of *Proteobacteria* can be explained by the high metabolic versatility (phototrophs, photoheterotrophs and chemolitotrophs) of this phylum (Kuykendall et al. 2005; Kersters et al. 2006). The classes *Cyanobacteria* and *Actinobacteria* appeared as the most abundant taxonomic groups and distinctively linked to the younger succession stages (Schmidt et al. 2008; Zumsteg et al. 2012; Rime et al. 2015). *Cyanobacteria* are thought to play a key role in barren soils close to glacier terminus, where there is notable nutrient scarcity (cf. Arróniz-Crespo et al. 2014), since they can incorporate C and N into topsoil layers through their phototrophic and diazotrophic activities and, consequently, promote later colonization (Kastovská et al. 2005; Schmidt et al. 2008; Frey et al. 2013). In contrast, *Actinobacteria* are able to decompose organic matter, including recalcitrant polymers (Heuer et al. 1997; Vetrovsky et al. 2014). The participation of *Actinobacteria* in the degradation of allochthonous organic matter (from aeolian or glacier stream deposits) or the organic matter that builds up under the ice sheet could be key to the subsequent colonization

of these oligotrophic soils since recalcitrant organic matter is converted into forms that are accessible to other microorganisms (Bardgett et al. 2007; Hawes 2008).

The changes observed in the composition of fungal communities along the chronosequences revealed a succession process likely influenced by their biotic interactions. *Chytridiomycetes*, *Lecanoromycetes* (lichen-forming ascomycetes) and *Tremellomycetes* were more abundant in the proximity of the glacier terminus than at later succession stages. The existence of *Chytridiomycetes* at these sites may be determined by the presence of *Cyanobacteria*, as they are common parasites of these microorganisms (Sonstebo and Rohrlack 2011; Sime-Ngando 2012; Gerphagnon et al. 2013). The presence of *Lecanoromycetes* could be related to the fact that lichen associations are key organisms in early succession stages, since they facilitate later colonization by other organisms, mainly through nutrient input to the ecosystem and soil stabilization (Brankatschk et al. 2011; de los Ríos et al. 2011). The concurrent presence of *Tremellomycetes* and *Lecanoromycetes* could be explained by the lichenicolous behaviour of many taxa within the former class (Millanes et al. 2011). Along the chronosequences, plant-associated fungi become increasingly represented. In fact, the high abundance of *Helotiales* (*Leotiomycetes*), which includes many species forming mycorrhizal associations with the plant family *Ericaceae* (Walker et al. 2011), coincided with the presence of *Empetrum rubrum* and *Gaultheria mucronata* in both forefields (Arróniz-Crespo et al. 2014). Fungal community compositions in succession stages dominated by vascular plants differed notably between the two chronosequences. In the SS forefield, fungal community dominance shifted across successions from Ascomycota to Basidiomycota (mainly through increase of *Agaricomycetes* abundance), similarly to prior observations in Alpine and North American glacier forefields (Jumpponen 2003; Zumsteg et al. 2012; Welc et al. 2014). This pattern was not, however, detected along the NS chronosequence mostly because of the dominance of *Archaeorhizomycetes* class (*Ascomycota*) in the early *Nothofagus* forest stages of this chronosequence (66 yrs since ice retreat). This ubiquitous soil class is generally found in roots and rhizospheres but without forming any recognizable mycorrhizal structure (Rosling et al. 2011). Previously reported correlations between *Archaeorhizomycetes* abundances and several habitat-related environmental variables (Rosling et al. 2013) suggest that this dominance is the outcome of differences in abiotic

factors between both forefields which could be related to the slower forest development.

Algae in glacier forefield chronosequences have been less investigated previously. The ubiquity of most algal taxa found in the two glacier forefields analysed in this study was consistent with the known widespread distribution of this taxonomic group in terrestrial environments (Broady 1996; Elster 2002; Büdel 2003; Rindi et al. 2010). Algal phylotypes do not necessarily indicate metabolic active forms, since they are able to remain dormant (Kastovská et al. 2005; Stibal et al. 2006; Lennon and Jones 2011). In fact, Kastovská et al. (2005) suggested the existence of a reservoir for algal propagules in previously ice-covered grounds. However, some algal orders could be associated to given succession stages in the analyzed forefields. The most common algal taxa found have been also observed in other deglaciated areas (Kastovská et al. 2005; Stibal et al. 2006). Among them, *Prasiola calophylla* showed a high abundance at sites close to the glacier terminus, stages to which this order has been previously described to be significantly associated (Broady 1989). Hence, its source is possibly the glacier ice, as this taxon has been detected on terminal walls of glaciers. On the other hand, representatives of the *Desmidiales* order were found to be much more abundant in the latest succession stages (especially in NS chronosequence), similarly to what has been previously described by Rasran (2004). In contrast, *Eudorina*, a widespread colonial genus belonging to the *Chlamydomonadales* (Schmidt et al. 2011), was highly represented in all succession stages except in the proximity of glacier terminus of both forefields. Interestingly, *Trebouxia*, genus commonly present in lichen symbioses (Friedl and Büdel, 2008), was broadly represented along each chronosequence. Distributions and abundances of this taxon did not match those of *Lecanoromycetes* (most abundant class of lichen-forming fungi) suggesting it could include free-living forms, as proposed for Antarctic lithobiontic communities (Yung et al. 2014) and those of other habitats (Bubrick et al. 1984).

Greater similarities in microbial composition were noted between the youngest succession stages of both chronosequences compared to subsequent stages. These results confirm that similar microorganisms colonized the recently deglaciated soils from both forefields, consistent with the findings of Skidmore et al. (2005) and Bajerski and Wagner (2013). Once succession started, abrupt changes in bacterial, fungal and

algal community composition took place, as it was revealed by the segregation of initial succession stages shown in the different ordinations and the high turnover rates detected between initial succession stages and immediately subsequent ones. However, high turnover rates, not associated to changes in diversity indices, were also detected later in the succession, indicating a replacement of taxa along the entire forefields. This pattern of succession could reflect an initial colonization by a pool of cosmopolitan, generalist microorganisms (Fierer et al. 2010; Frey et al. 2013), which may have remained under the ice or arose from different origins, such as the ice sheet itself or adjacent soil areas by aeolian deposition (Hodson et al. 2008; Takeuchi 2011). As succession progresses, pioneer microorganisms are substituted by more specialized taxa (Fierer et al. 2007ab; Frey et al. 2013). The increased complexity of soil structure and the local effects induced by the appearance of plants along a chronosequence could determine a greater effect of habitat filtering on microbial community assembly (Bardgett and Walker 2004; Horner-Devine and Bohannan 2006; Knelman et al. 2012; Brown and Jumpponen 2014).

Bacterial communities in both glacier forefields were richer and more diverse than fungal and algal communities (*c.* 5:3:2 *H'* values). This concurs with figures reported for deglaciated territories in the Northern Hemisphere (Zumsteg et al. 2012; Brown and Jumpponen 2014) and representative soils in dominant ecosystem types (Fierer et al. 2007b). Our joint analysis of bacterial, fungal and algal communities revealed differences in the succession trajectories of the three groups of microorganisms. Hence, while algae seemed to be mostly randomly distributed, a higher influence of succession stage on OTU distribution was detected for bacterial and fungal communities, as it was depicted by the high abundance of unique OTUs found at every succession stage. Moreover, the ubiquity of algae also suggests that they may not be so important drivers of succession as bacteria and fungi. In initial stages of succession, bacteria seem to play a dominant role over fungi. In effect, *Cyanobacteria* and *Actinobacteria* (specific to the youngest succession stages examined here) have been described as essential to the colonization of plant-free soils close to glacier termini (Heuer et al, 1997; Kastovská et al. 2005; Schmidt et al. 2008; Zumsteg et al. 2012; Frey et al. 2013; Vetrovsky et al. 2014). In contrast, the roles of fungi could be more relevant as succession progress and nutrient are accessible, since they highly depend on the availability of carbon and nitrogen.

Therefore, our data point to a more dynamic community composition and more dependent on biotic interactions for fungi than for bacteria along these Tierra del Fuego glacier chronosequences. Higher habitat requirements and more constraints for their dispersal have been also described for fungi compared to bacteria in other geographic areas, especially in early succession stages (Brown and Jumpponen 2014).

Microbial turnover rates per year in these Tierra del Fuego glacier forefields (values from 1.7% to 35.4%) were faster than those reported for bacteria in the high Arctic zone (0.7% to 7%) (Schutte et al. 2010) and Central Asia (0.9% to 19%) (Wu et al. 2012). We propose the mild oceanic climate conditions of Tierra del Fuego as the main factor responsible for faster biological primary succession in these southern hemisphere forefields.

The distinct temporal dynamics of microbial succession in the NS and SS forefields observed here points to factors besides soil surface age driving primary succession. Primary succession is influenced by local and regional differences in abiotic factors, such as the precipitation regime, geographical orientation, sunlight exposure, soil pH and organic compounds (Sigler and Zeyer 2002; Wang et al. 2010; Philippot et al. 2011; Bajerski and Wagner 2013). Thus, the differences in climate conditions between both aspects of the Cordillera Darwin (Holmlund and Fuenzalida 1995) affect microbial community assembly in the studied forefields. Accordingly, fast rates of plant succession and soil development (Arróniz-Crespo et al. 2014) as well as a high diversity of endosymbiotic microorganisms of the *Nostoc* genus in *Gunnera magellanica* (Fernández-Martínez et al. 2013) have been attributed to such climate differences. The composition and activity of established soil microbial communities is also affected by the precipitation regime (Zeglin et al. 2013).

Our findings suggest that the different plant succession observed in both forefields was preceded by distinct microbial succession dynamics. Hence, the faster plant colonization detected in the SS glacier forefield is likely the consequence of earlier effects of microbial metabolic activities and competition with higher plants (Sigler and Zeyer 2002; Miniaci et al. 2007; Knelman et al. 2012; Rime et al. 2015), thus multiplying the direct effects of climatic factors on plant succession.

1.5 Supporting information

Supporting information 1.1. PCR specifications

Primers for each reaction included an artificial tail (universal consensus sequence tags, CS1 and CS2). For all three reactions, 50 ng of purified DNA (using QiaEx II Purification Kit, Quiagen Laboratories Inc.) were introduced as template and amplification was carried out with FastStart High Fidelity Enzyme (Roche Applied Science Ltd.).

Bacterial PCR of the *16S* rDNA V1-V3 gene region was carried out following the C0t protocol (Fluidigm: Access array system™ user guide v.3, 2010, http://www.fluidigm.com/user-document-request.htm).

Fungal PCR of internal transcribed spacers (ITS) 1 and 2 and *5.8S* ribosomal RNA was carried out at an annealing temperature of 52° C and 30 cycles of amplification.

Algal RuBisCo *rbc*L gene fragment amplification was performed following a touch-down PCR with a variable annealing temperature for 5 cycles, descending 1.5° C per cycle (from 52° C to 46° C) and for 27 cycles at 45° C.

Supporting information 1.2. Second PCRs and pyrosequencing specifications

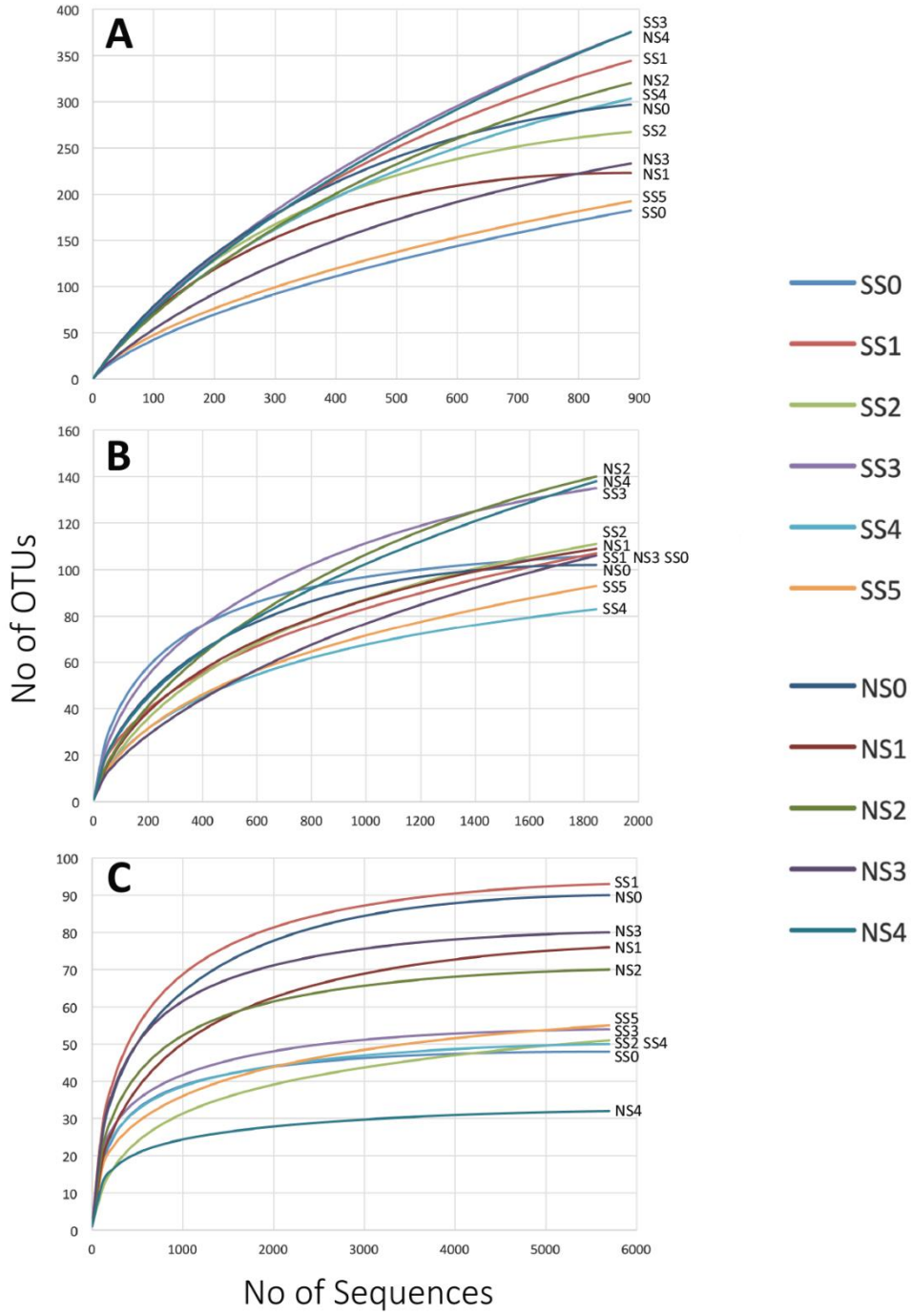
Once the first amplifications were conducted using primers containing universal consensus tags (CS1 and CS2), a nested PCR was carried out using the purified products of these previous PCRs as templates and two primers targeting CS1 and CS2, modified towards the 3' end to contain the pyrosequencing adaptors A and B and a 10 bp multiple identifier barcode (MIDs) specific to each sampling point. The nested PCR reactions were set up for an annealing temperature of 60°C and 8 cycles of amplification. DNA concentrations of different PCR products were estimated using a fluorescence method (PicoGreen assay, Invitrogen™). Next, the three obtained products of amplification of bacterial, fungal and algal chromosomal regions for the same sampling point were pooled at equimolar concentrations and purified using Agencourt XP Ampure Beads (Beckman Coulter Inc.). Copy numbers of each genomic region were estimated by qPCR using the 454 titanium DNA standards primer premix kit and KAPA SYBR FAST qPCR kit for LC480 as mastermix (KAPA Biosystems). The final step prior to pyrosequencing consisted of an emulsion PCR to couple 0.08 copies of each amplified region to each

bead, followed by a final load of 1,580,000 beads introduced into half the picotitre plate. Pyrosequencing was carried out from the forward primer-end for bacteria and algae and from the reverse primer-end for fungi using the Roche 454 GS FLX Titanium (Roche Applied Science) platform.

Supporting information Table 1.1. Shared bacterial, fungal and algal OTUs (expressed as a percentage of the total number of OTUs from rarefied dataset) in each succession stage versus the remaining stages in the same glacier forefield or versus all the succession stages in its counterpart glacier forefield.

Succession stage	OTUs shared with	Bacteria	Fungi	Algae
SS0	SS	9.40	17.86	42.28
	NS	23.50	17.86	53.55
SS1	SS	26.35	31.08	45.77
	NS	23.95	26.64	48.72
SS2	SS	32.61	30.01	48.68
	NS	25.09	20.33	49.81
SS3	SS	30.31	28.09	47.33
	NS	25.69	17.03	51.63
SS4	SS	31.88	43.48	49.37
	NS	33.72	31.52	49.37
SS5	SS	28.41	18.15	44.81
	NS	41.79	32.67	53.29
NS0	SS	27.21	25.34	52.90
	NS	10.89	24.28	41.14
NS1	SS	34.24	42.82	48.84
	NS	32.76	27.59	48.84
NS2	SS	33.15	16.28	48.75
	NS	29.15	30.23	50.38
NS3	SS	37.58	20.04	48.55
	NS	36.32	32.44	48.55
NS4	SS	27.03	15.34	47.13
	NS	27.67	22.61	52.37

Supporting information Fig. 1.1.



Supporting information Fig. 1.1. Sequencing depth analyses of rarefied bacterial **(a)**, fungal **(b)** and algal **(c)** communities along the SS and NS chronosequences. Rarefaction curves show the number of expected OTUs (vertical axes), as determined by the analytical algorithm described by Chao et al. (2013) and Hsieh et al. (2013), plotted *versus* the rarefied number of sequences analysed at each sampling site (horizontal axes). Sites/succession stages (SS0-SS5 and NS0-NS4) are indicated by the sae colours in the three figures. Note the difference in scale in the major taxa plots.

CAPÍTULO 2

Ecología funcional de las comunidades microbianas del suelo a lo largo de zonas de retroceso de un glaciar en Tierra del Fuego (Chile)

Resumen

Una cronosecuencia establecida previamente en el Glaciar Pía (Tierra del Fuego, Chile), la cual presenta suelos de diferentes edades, se usó como marco de trabajo para analizar si el desarrollo sucesional de las comunidades microbianas está acompañado de cambios en las capacidades funcionales microbianas. Para comprobar esta hipótesis se utilizó el *microarray* funcional GeoChip 4.0 con el objeto de identificar la diversidad de los genes involucrados en actividades microbianas implicadas en los ciclos del carbono y del nitrógeno, así como otros genes relacionados con respuesta microbiana al estrés y con interacciones bióticas. Los cambios en las capacidades funcionales reflejaron, por lo general, las variaciones en composición taxonómica microbiana en cada etapa de sucesión. Se observaron además cambios importantes en los procesos de fijación de carbono y en procesos catabólicos, así como variaciones remarcables en el metabolismo del nitrógeno. En las etapas iniciales, los microorganismos parecen estar involucrados principalmente en rutas metabólicas que ayudan a incrementar la disponibilidad de nutrientes en el suelo, mientras que otras transformaciones microbianas, tales como desnitrificación y metanogénesis o degradación de sustratos orgánicos complejos, parecen presentar una mayor importancia en etapas más avanzadas. Los cambios encontrados en las poblaciones de virus reflejaron a su vez cambios en la diversidad microbiana. Por el contrario, las rutas metabólicas de respuesta al estrés aparecieron relativamente igual representadas en todas las comunidades a lo largo de la cronosecuencia. Por todo ello concluimos que la utilización de los nutrientes es probablemente la responsable principal de los cambios sucesionales de las comunidades microbianas en estos suelos.

Abstract

A previously established chronosequence from Pia Glacier forefield in Tierra del Fuego (Chile) containing soils of different age is used as framework to postulate that microbial successional development along this chronosequence would be accompanied by changes in functionality. To test this, the GeoChip 4.0 functional microarray was used to identify diversity of genes involved in microbial carbon and nitrogen metabolism, as well as other genes related to microbial stress response and biotic interactions. Changes

in putative functionality generally reflected succession-related taxonomic composition of soil microbiota. Major shifts in carbon fixation and catabolism were observed, as well as major changes in nitrogen metabolism. At initial microbial dominated succession stages, microorganisms could be mainly involved in pathways that help to increase nutrient availability, while more complex microbial transformations such as denitrification and methanogenesis and later degradation of complex organic substrates, could play more important roles at vegetated successional stages. Shifts in viral populations broadly reflected changes in microbial diversity. Conversely, stress response pathways appeared relatively well conserved for communities along the entire chronosequence. We conclude nutrient utilization is likely the major driver of microbial succession in these soils.

2.1 Introduction

Microorganisms play a fundamental role in the initial colonization of exposed soils after glacial retreat (Kandeler et al. 2006; Zumsteg et al. 2012; Brown and Jumpponen 2014; Rime et al. 2015). Pioneer microorganisms are responsible for most biological transformations and drive the development of stable and labile pools of nutrients (Borin et al. 2010; Bernasconi et al. 2011; Lapanje et al. 2012; Smittenberg et al. 2012) that facilitate the subsequent establishment of other microorganisms, and subsequently lichens, bryophytes and vascular plants (Grubb 1986; Sigler and Zeyer 2002; Jumpponen 2003; de los Ríos et al. 2011; Brown and Jumpponen 2014). Soil microbial communities underpin carbon (C) and nitrogen (N) transformation processes (*e.g.* photosynthesis, N_2 fixation, substrate decomposition, nutrient mineralization), which are essential for soil development and nutrient cycling in soils (Frenot et al. 1995, 1998; Zumsteg et al. 2012; Schulz et al. 2013; Welc et al. 2014).

In newly exposed glacier forefield soils organic carbon levels are low and limiting to microbial growth (Schulz et al. 2013). In these soils, microbial carbon input is primarily mediated by photosynthetic carbon fixation (Nemergut et al. 2007; Zumsteg et al. 2012; Frey et al. 2013) but autochthonous or allochthonous organic matter breakdown can be also an important source of nutrients (Hodkinson et al. 2003; Bardgett et al. 2007; Brankatschk et al. 2011; Guelland et al. 2013). The balance between autotrophic and heterotrophic processes at initial stages of colonization has been suggested as a key factor in shaping the overall forefield development and the regulation of the associated biogeochemical systems (Fierer et al. 2010; Schulz et al. 2013; Bradley et al. 2014). Soil microbial communities are also involved in other important carbon transformations along glacier forefield soils such as methanogenesis and methane oxidation (Murrel and Jetten 2009; Bárcena et al. 2011; Nauer et al. 2012; Hofmann et al. 2013).

Nitrogen has been identified as the main limiting nutrient in high latitude ecosystems and also possibly a key factor in the regulation of forefield ecosystem functional dynamics (Shaw and Harte 2001; Zielke et al. 2005; Bradley et al. 2014; Thébault et al. 2014). At initial stages of soil development the main microbial contribution is atmospheric N_2 fixation, either by free-living or symbiotic diazotrophs (Raggio et al. 2012; Arróniz-Crespo et al. 2014; Bradley et al. 2014). Nitrogen can also be released from ancient autochthonous or allochthonous nitrogen-rich organic matter (*i.e.*

chitinolysis and proteolysis) by heterotrophic or mixotrophic microbial degradation (Duc et al. 2009; Sattin et al. 2009; Brankatschk et al. 2011; Schulz et al. 2013). During successional development of vegetation the contribution of microorganisms to nitrogen cycling becomes more related to the transformation of nitrogenized compounds from freshly deposited organic matter (Ollivier et al. 2011). Microbially mediated ammonification and nitrification lead to increases in bioavailable nitrogen (Deiglmayr et al. 2006; Kandeler et al. 2006; Hahn et al. 2013). Nitrogen may also be lost to the soil system due to nitrogen volatilization, via microbial denitrification, as well as leaching of mobile nitrates to deeper soil layers (Brankatschk et al. 2011; Ollivier et al. 2011; Schulz et al. 2013).

Soil microbial communities are also heavily influenced by edaphic factors (Lauber et al. 2009; Griffiths et al. 2011; Roy et al. 2013; Tedersoo et al. 2014). The progressive changes in pH, moisture and nutrient availability that follow glacial retreat shape the community structure along the primary succession (Sigler et al. 2002; Sigler and Zeyer 2004; Arróniz-Crespo et al. 2014; Meola et al. 2014). While some microorganisms can acclimate to shifts in abiotic factors, other taxa do not and are consequently replaced (Sigler and Zeyer 2004; Lozupone and Knight 2007; Schimel et al. 2007; Fierer et al. 2010; Meola et al. 2014). Microbial succession also involves biotic interactions that induce complicated network structures (Cong et al. 2015). The competition for nutrient resources and space among different microbial groups can be regulated by synthesis of antibiotics along the succession (Sigler and Zeyer 2004; Hibbing et al. 2009) and therefore, the detection of antibiotic resistance genes can be interpreted as the result of interspecific microbial interactions (Chan et al. 2013; Wei et al. 2015b). The existence of viruses (particularly phage) can be considered another potential biotic driver in primary succession, as they can exert a bottom-up regulatory effect on microbial communities (Breitbart et al. 2005; Koskella and Brockhurst 2014; Wei et al. 2015b).

The Pia Glacier forefield is located at the southern slope of Cordillera Darwin (Tierra del Fuego, Chile). Cordillera Darwin presents *c*. 80% of the surface covered by an ice cap, although most of the glaciers located at the mountain range have been receding constantly since the Little Ice Age (*c*. between AD 1750 and 1850) (Masiokas et al. 2009). The area has a cool maritime climate with an average 5° C of temperature with little seasonal variations (Molina 1983; Burgos 1985; Santana et al. 2006). The southern

slopes of Cordillera Darwin receive heavy rainfall of *c.* 1600 mm/year (Holmlund and Fuenzalida 1995; Santana et al. 2006; Koppes et al. 2009; López et al. 2010). This forefield presents a clear sequence of moraine bands and rapid rates of vegetation growth and soil development, with *Nothofagus* tree-dominated stages present after only 34 years of soil surface exposure (Table 2.1, Sancho et al. 2011; Arróniz-Crespo et al. 2014). The vegetation pattern along the chronosequence is characterized by pioneer lichens (*Placopsis* spp. and *Sterocaulum* sp.) and mosses (e.g. *Ditrichum cylindricarpum*) settled in soils ice-free for 4 to 7 yrs, along with herbs (*Gunnera magellanica*, *Uncinia tenuis*) and, subsequently, bushes (*Gaultheria mucronata*, *Empetrum rubrum*) and young *Nothofagus antarctica* and *N*. *betuloides* at soils ice-free for 10 to 19 yrs and the development of *Nothofagus* forest at soils ice-free for more than 34 yrs (Table 2.1, see Fig. B.4, page 50).

Soils located close to the glacier front (from 1 to 7 yrs being ice-free) are characterized by high pH, very low or undetectable total C and N contents, and low concentration of extractable NH_4^+ – N but relatively high concentration of NO_3^- – N (around 10 mg kg^{-1} after 4 and 7 years being ice-free, Table 1). After 10 years of soil exposure, significant accumulation of N, C and NH_4^+ – N occurs and soil development progresses rapidly to an organic soil within the forest, which presents high contents of C and N (over 39% TC and 1.5% TN), NH_4^+ – N (88.5 mg kg^{-1}), NO_3^- – N (46.5 mg kg^{-1}) and low pH (4.5) (Table 2.1). More detailed soil chemical properties along the chronosequence have been described in Arróniz-Crespo et al. (2014).

We hypothesized that this rapid succession must be due, at least in part, to microbial conditioning nutrient cycling in soil via carbon and nitrogen transformations, and that succession likely involves overcoming significant abiotic and biotic stressors. We analyzed the functional gene profile of microbial communities using the GeoChip 4 micro-array in order to target key gene markers for major metabolic, stress response pathways and interactions (He et al. 2007; Yergeau et al. 2007; Chan et al. 2013; Tu et al. 2014; Yue et al. 2015). This study contributes novel insight to microbial functional ecology in a well-defined soil chronosequence.

Table 2.1. Site vegetation description and environmental attributes measures along the chronosequence of Pia Glacier forefield. Only mean values obtained for the three sampled transects are represented. Data were obtained from Arróniz-Crespo et al. (2014).

SAMPLING POINT (SOIL SURFACE AGE)	Vegetation	pH	NH_4	NO_2	NO_3	Inorganic N	% total N	% total C
1 yr	Bare soil	6.81±0.34	0	0	1.82±0.63	1.82±0.63	0	0.25±0.03
4 yrs	Pioneer lichens (*Sterocaulon sp.*) and mosses (*Ditrichium cylindricarpum*, *Acroschisma wilsonii*)	6.72±0.14	1.04±0	0	12.38±5.07	13.42±4.4	0	0.24±0.025
7 yrs	Lichens (*Sterocaulon sp.*) and pioneer herbs (*Gunnera magellanica*)	5.72±0.18	5.27±2.29	0	10.12±0	12.52±2.65	0.02±0.012	0.78±0.27
10 yrs	Herbs (*Gunnera magellanica*, *Uncinia tenuis*, *Gaultheria mucronata*, *Empetrum rubrum* and young *Nothofagus spp.*)	5.17±0.2	30.22±10	0	4.62±1.43	34.84±9.88	0.03±0.015	1.7±0.34
19 yrs	Herbs and bushes (*Uncinia tenuis*, *Gaultheria mucronata*, *Empetrum rubrum*, young *Nothofagus antarctica* and *N. betuloides*)	5.04±0.46	37.7±9.9	0.15±0	1.65±0.05	39.5±10.72	0.22±0.11	6.61±2.81
34 yrs	Forest (*Nothofagus antarctica*, *N. betuloides*)	4.55±0.02	88.5±1.3	0.13±0	46.46±31.5	135.09±30.21	1.51±0.098	39.03±1.34

* NH_4^+, NO_2^-, NO_3^- and Inorganic N are expressed in mg/kg of soil.

2.2 Materials and Methods

The study was conducted along a chronosequence established in soils from the Pia Glacier forefield (54° 46' S 69° 40' W) of ice-free times ranging from 1 to 34 years, attributed using aerial photographs, dendrochronology and lichenometry (Sancho et al. 2011; Arróniz-Crespo et al. 2014). Samples from different succession stages were collected during the austral summer of 2009 at sites that have been ice-free for 1, 4, 7, 10, 19 and 34 years (Table 2.1). At each succession stage, 3 sampling points were selected at 3 parallel transects established along the glacier forefield, from the glacier terminus towards the oldest dated moraines. At each location, three surface soil samples (*c.* 0-5 cm depth) each 1m apart were aseptically recovered and pooled to yield a 200g composite sample for each location. Samples were directly frozen and stored at -20°C until processed.

Genomic DNA was extracted using the PowerMax Soil DNA Isolation Kit (MO BIO Laboratories, Inc.). DNA concentrations were determined using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific™). DNA samples from the same succession stage (same soil surface age) were pooled in equimolar concentrations for further analysis. Pooled DNA samples were then concentrated using a SpeedVac concentrator (Savant Inc.) and purified using QiaEX II DNA Purification Kit (Qiagen Laboratories INC.).

Functional diversity was assessed using the GeoChip 4.0 microarray. This comprises 84,000 50-mer oligonucleotide probes covering 141,995 gene variants from 410 distinct functional gene families involved in microbial carbon, nitrogen, sulphur, and phosphorus cycling, energy metabolism, antibiotic resistance, metal resistance/reduction, organic remediation, stress responses, bacteriophage and virulence, major biogeochemical, ecological and other metabolic processes (Tu et al. 2014). The GeoChip hybridization was carried out according to manufacturer's instructions as previously described (Zhou et al. 2008). The normalized hybridization output data were reorganized on the basis of functional categories (in this study C and N cycle, stress responses, viral diversity and antibiotic resistance gene signatures) as previously described (Chan et al. 2013; Wei et al. 2015a). Pathway-specific GeoChip oligonucleotides are presented in a very large number in the analysis, creating a level of redundancy that allows a high degree of confidence in signal recovery, inferring occurrence of any given pathway (He et al. 2007).

Contributions of different taxa to each metabolic pathway (C and N cycles, Supporting information Table 2.1 and 2.2) and stress responses were depicted using a heat map, where signal intensity was used as a proxy for relative abundance. Additionally, radar charts were constructed for gene data involved in C and N cycles among different sampling points and phyla. Probes that returned positive signals from all of the sampling points were defined as 'ubiquitous' genes while those with positive signals at only one sampling point were defined as 'endemic' genes. Hybridization of DNA from sampling points along the chronosequence was achieved with 94.56% of the probes on average, over a total 83,992 probes. The GeoChip dataset reported in this paper is publicly available at http://ieg.ou.edu/4download/.

Bray-Curtis similarities for functional genes among different samples were calculated and visualised using non-metric multidimensional scaling (NMDS) with R package vegan v. 2.2-1 (Oksanen et al. 2013). This was applied to all pathways except anammox, which was excluded from this analysis due to their occurrence in a single phylum (*Planctomycetes*).

2.3 Results

Functional community structure

Among the 79,419 probes returning positive signals, 13,000 were derived from genes involved in carbon (C) cycle, 3,428 from nitrogen (N) cycle, and 12,471 from stress responses genes, while 907 corresponded to viral signatures and 260 to antibiotic resistance genes. The microarray analysis revealed that communities from all the succession stages supported potential for autotrophic, heterotrophic, diazotrophic and stress response pathways (Fig. 2.1, 2.2). The most taxonomically widespread functional genes were those involved in degradation of organic polymers (Fig. 2.1), while the most group-specific genes were related to anaerobic ammonium oxidation (anammox) (Fig. 2.1). The *Euryarchaeota* and *Proteobacteria* showed the greatest metabolic diversity (Fig. 2.1).

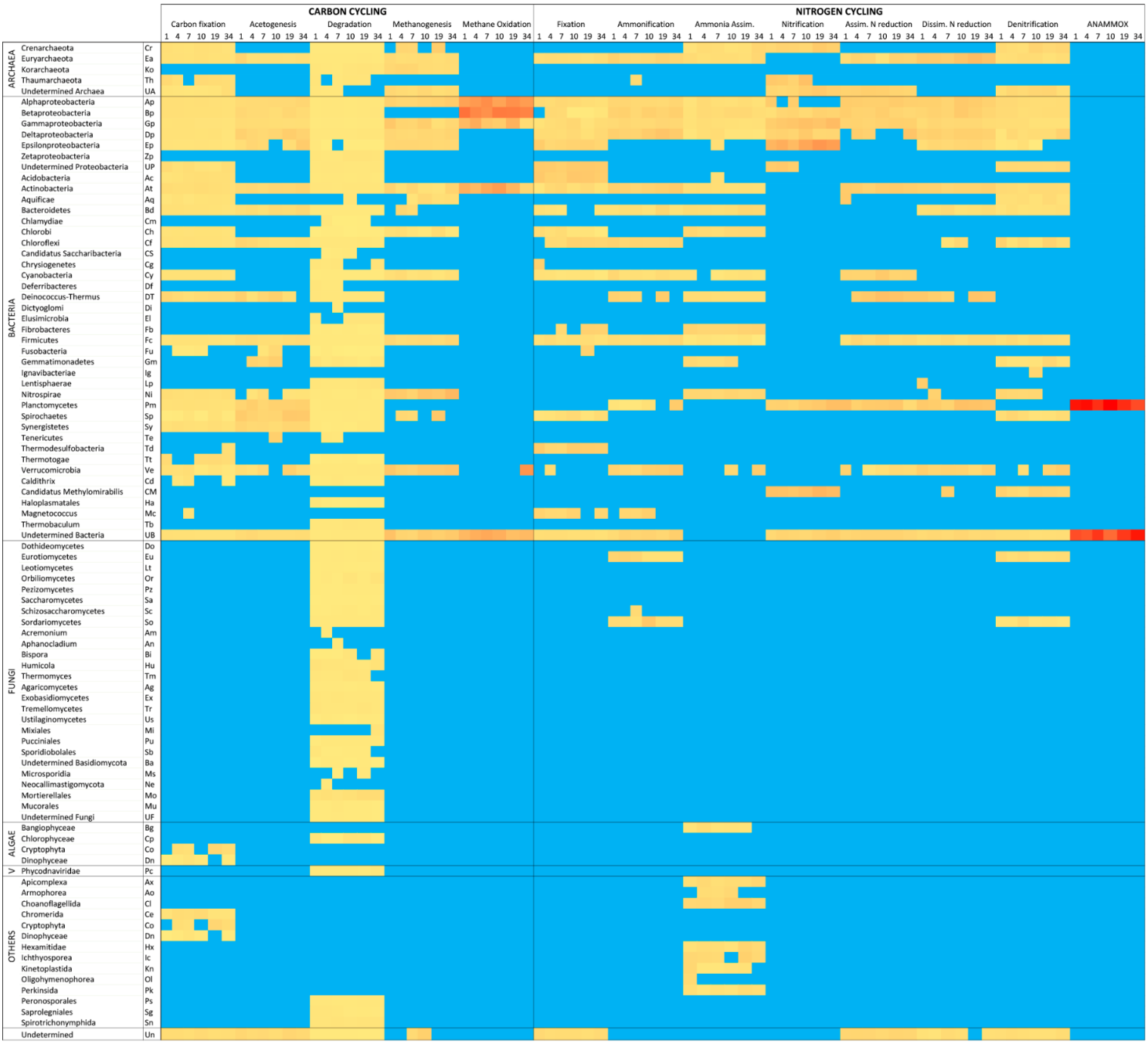


Fig. 2.1. Distribution heatmap of carbon and nitrogen cycling genes among microbial taxa and succession stages. *Blue* color indicates non-detected signal intensity, while positive signals were indicated from *yellow* color (lower values of signal intensity) to *red* color (higher levels of signal intensity).

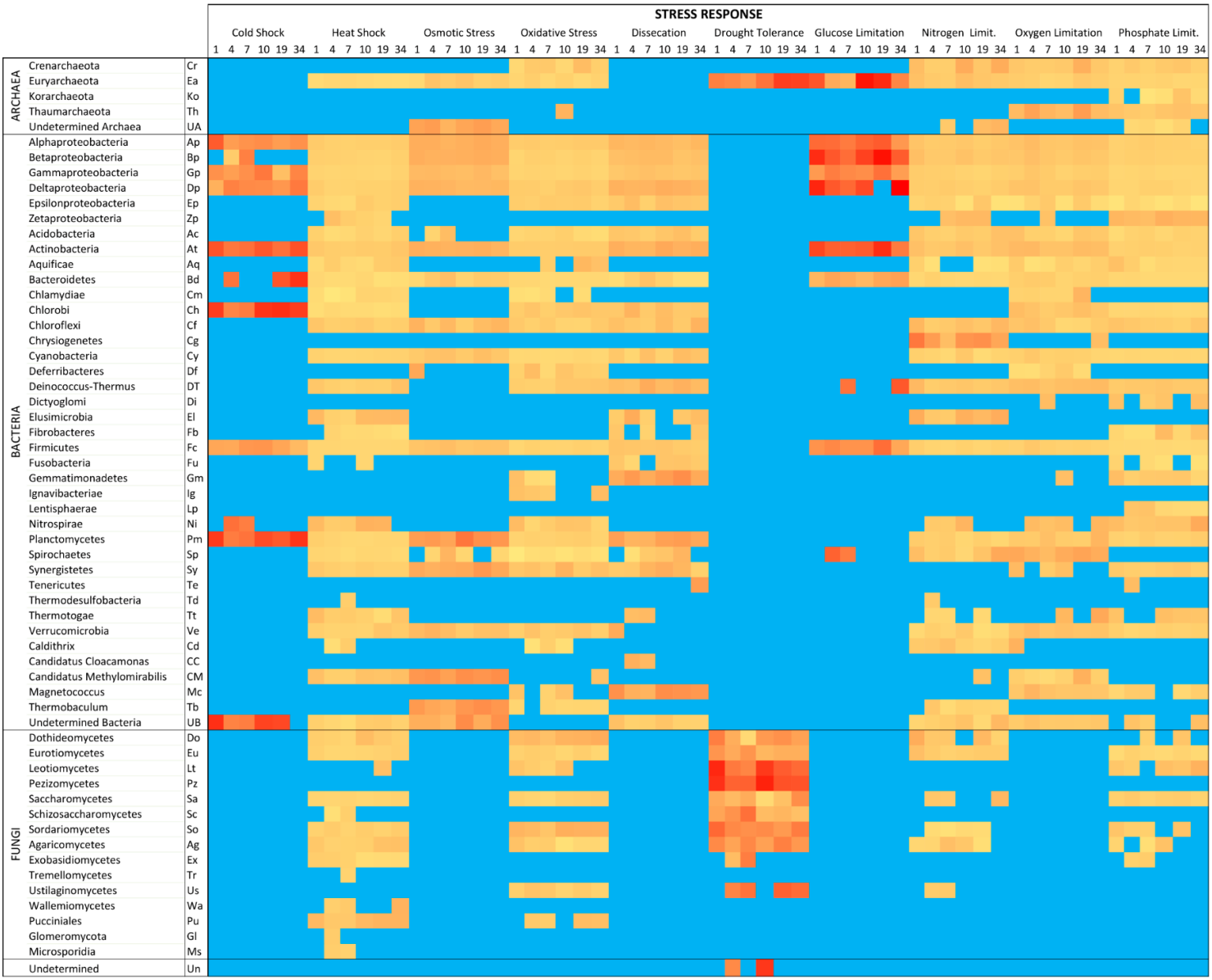


Fig. 2.2. Distribution heatmap of stress reponse genes among microbial taxa and succession stages. *Blue* color indicates non-detected signal intensity, while positive signals were indicated from *yellow* color (lower values of signal intensity) to *red* color (higher levels of signal intensity).

Endemic (present only in one succession stage) and ubiquitous (present in all successional stages) genes accounted for 22.19% and 36.16% of total detected genes respectively (Supporting information Fig. 2.1). Bacteria displayed the highest abundance of endemic (19.23%) and ubiquitous (32.73%) genes, but the lowest ratio of endemic to

ubiquitous genes (Table 2.2a). C and N cycle and stress response pathways showed similar levels of endemic and ubiquitous genes abundance (Table 2.2b). The abundance of endemic genes for all major taxa and metabolic pathways was greater in soils ice-free for 7 years than at all other successional stages (Table 2.2a-b).

Table 2.2. Ubiquitous (U, present in all the sampling points) and endemic (E, present only in one sampling point) genes. **(a)** Comparison among different taxa of endemic to ubiquitous genes ratio and percentages of endemic and ubiquitous genes total and for the different succession stages. **(b)** Comparison among different pathways of endemic to ubiquitous genes ratio and percentages of total endemic and ubiquitous genes total and for the different succession stages.

A

TAXA	E/U (total)	U (total) %	E (total) %	E (1yr) %	E (4yr) %	E (7yr) %	E (10yr) %	E (19yr) %	E (34yr) %
Archaea	0.89	0.97	0.86	0.12	0.19	0.31	0.10	0.07	0.07
Bacteria	0.58	32.73	19.23	2.47	3.87	7.62	1.97	1.65	1.64
Fungi	0.91	1.99	1.81	0.25	0.35	0.67	0.20	0.18	0.17
Others	0.62	0.47	0.29	0.06	0.05	0.10	0.03	0.02	0.03

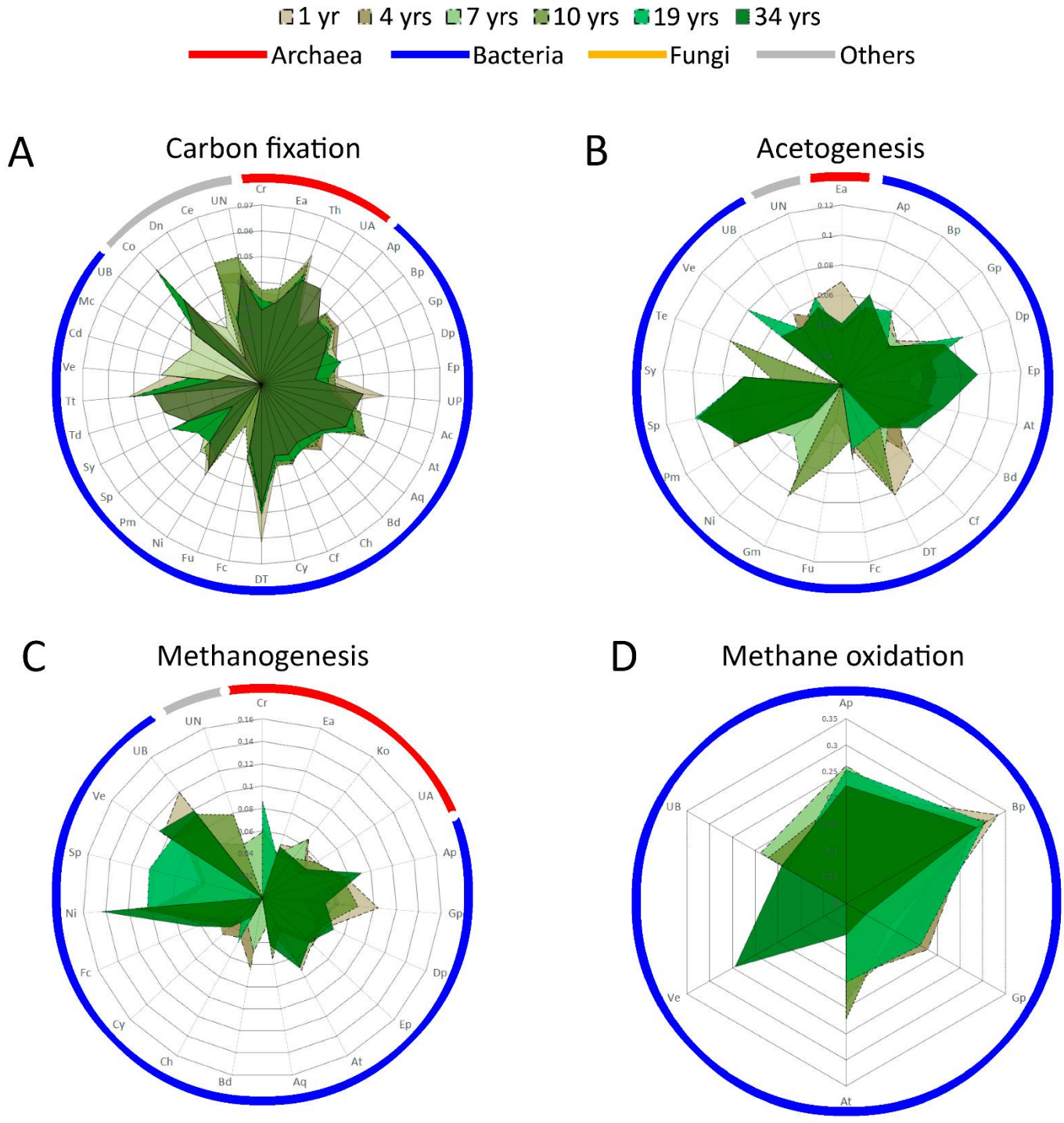
B

METABOLIC CATEGORY	E/U (total)	U (total) %	E (total) %	E (1yr) %	E (4yr) %	E (7yr) %	E (10yr) %	E (19yr) %	E (34yr) %
C cycling	0.59	36.84	21.78	2.91	4.36	8.30	2.30	1.97	1.94
N cycling	0.65	34.10	22.05	2.95	4.43	8.69	2.28	1.95	1.75
Stress resp.	0.63	36.02	26.67	2.87	4.55	9.14	2.31	1.86	1.94

Carbon metabolism

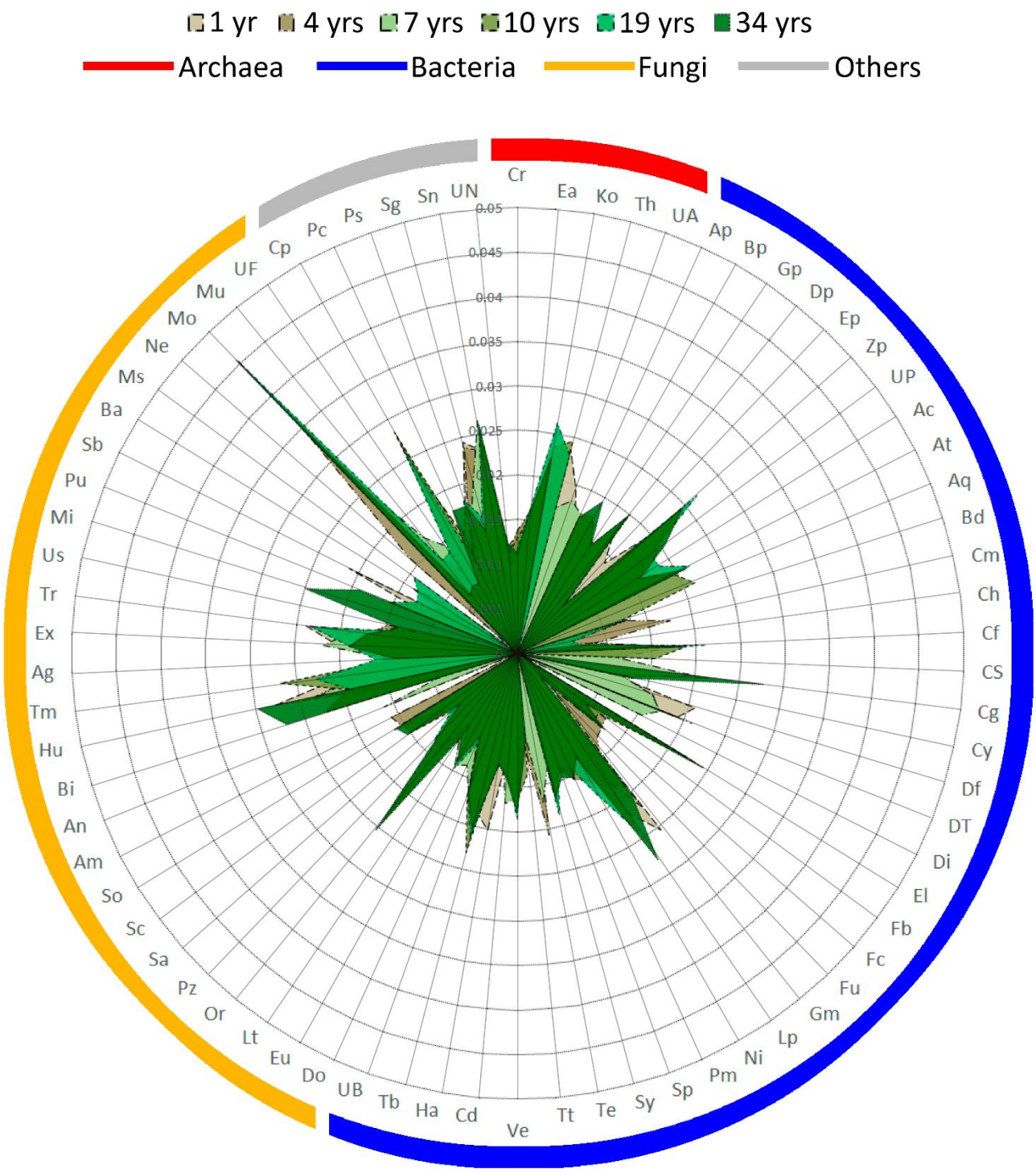
Autotrophy, acetogenesis, methanogenesis, methane oxidation and organic compound degradation genes were detected for microbial communities across all succession stages (Fig. 2.1, 2.3, 2.4). The potential for carbon fixation (photoautotrophy and chemoautotrophy) was indicated among 34 taxa among archaea, bacteria and algae (Fig. 2.1, 2.3a). Potential for acetogenesis was found in the *Euryarcheota* and 20 bacterial taxa with strongest signals for *Deltaproteobacteria*, *Epsilonproteobacteria* and *Sphirochaetes* (Fig. 2.1, 2.3b). The potential for methanogenesis was found in 4 archaeal and 15 bacterial taxa, with stronger signals for bacterial taxa (Fig. 2.1, 2.3c). We identified 6 bacterial taxa with capacity for methane oxidation. The strongest signal was for *Proteobacteria* (especially for *Betaproteobacteria*) and these were evenly distributed

along the chronosequence. *Verrucomicrobia* methane oxidation genes were detected only in soils that were ice-free for 34 yrs (Fig. 2.1, 2.3d). The ability to carry out degradation of different organic compounds (*e.g.* starch, chitin, cellulose, lignin, pectin) was identified in 74 taxa among all the major domains (Fig. 2.1, 2.4) with stronger signals for fungal taxa at different succession stages.



◀**Fig. 2.3.** Radar charts depicting taxa-function relationships for the C cycling genes. Relative signal intensity was normalized by the number of probes per taxon and expressed in percentage of total activity. Values from each sampling point were plotted for the carbon cycling genes involved in **(a)** Carbon fixation; **(b)** Acetogenesis; **(c)** Methanogenesis and **(d)** Methane Oxidation. Two-character codes denote microbial phyla as follows: Archaea: Cr: *Crenarchaeota*, Ea: *Euryarchaeota*, Ko: *Korarchaeota*, Th: *Thaumarchaeota*, UA: Undetermined Archaea. Bacteria: Ap: *Alphaproteobacteria*, Bp: *Betaproteobacteria*, Gp: *Gammaproteobacteria*, Dp: *Deltaproteobacteria*, Ep: *Epsilonproteobacteria*, Zp: *Zetaproteobacteria*, UP: Undetermined *Proteobacteria*, Ac: *Acidobacteria*, At: *Actinobacteria*, Aq: *Aquificae*, Bd: *Bacteroidetes*, Cm: *Chlamydiae*, Ch: *Chlorobi*, Cf: *Chloroflexi*, CS: *Candidatus Saccharibacteria*, Cg: *Chrysiogenetes*, Cy: *Cyanobacteria*, Df: *Deferribacteres*, DT: *Deinococcus-Thermus*, Di: *Dictyoglomi*, El: *Elusimicrobia*, Fb: *Fibrobacteres*, Fc: *Firmicutes*, Fu: *Fusobacteria*, Gm: *Gemmatimonadetes*, Ig: *Ignavibacteriae*, Lp: *Lentisphaerae*, Ni: *Nitrospirae*, Pm: *Planctomycetes*, Sp: *Spirochaetes*, Sy: *Synergistetes*, Te: *Tenericutes*, Td: *Thermodesulfobacteria*, Tt: *Thermotogae*, Ve: *Verrucomicrobia*, Cd: *Calditrix*, Ha: *Haloplasmatales*, Mc: *Magnetococcus*, Tb: *Thermobaculum*, UB: Undetermined Bacteria. Fungi: Do: *Dothideomycetes*, Eu: *Eurotiomycetes*, Lt: *Leotiomycetes*, Or: *Orbiliomycetes*, Pz: *Pezizomycetes*, Sa: *Saccharomycetes*, Sc: *Schizosaccharomycetes*, So: *Sordariomycetes*, Am: *Acremonium*, An: *Aphanoclauidim*, Bi: *Bispora*, Hu: *Humicola*, Tm: *Thermomyces*, Ag: *Agaricomycetes*, Ex: *Exobasidiomycetes*, Tr: *Tremellomycetes*, Us: *Ustilaginomycetes*, Mi: *Mixiales*, Pu: *Pucciniales*, Sb: *Sporidiobolales*, Ba: Undetermined *Basidiomycota*, Ms: *Microsporidia*, Ne: *Neocallimastigomycota*, Mo: *Morteriellales*, Mu: *Mucorales*, UF: Undetermined Fungi. Others: Cp: *Chlorophyceae*, Co: *Cryptophyta*, Dn: *Dinophyceae*, Pc: *Phycodnaviridae*, Ce: *Chromerida*, Ps: *Peronosporales*, Sg: *Saprolegniales*, Sn: *Spirotrichonimphida*. Un: Undetermined.

Fig. 2.4. Radar charts depicting taxa-function relationships for Carbon degradation genes. Relative signal intensity was normalized by the number of probes per taxon and expressed in percentage of total activity. Two-character codes denote microbial phyla (see Figure 2.3). ▶



Some of the major pathways involved in C cycle were associated to specific succession stages along the glacier chronosequence (Fig. 2.5). Degradative pathways for different organic polymers (starch, pectin, lignin and chitin) were associated to microbial communities from soils being ice-free for 1 yr. Meanwhile, the potential for cellulose

degradation was associated to soils being ice-free for 1 yr but also closely positioned to soils being ice-free for 34 yrs. Glyoxylate cycle and different degradative pathways (*e.g.* degradation of terpenes, cutine and hemicellulose) were associated to soils being ice-free for 19 yrs and aromatic compounds transformation to soils being ice-free for 34 yrs. The distribution of C-1 pathways genes ('CH_4 cycle') was also not homogeneous: while potential for methanogenesis was associated to microbial communities from soils being ice-free for 7 yrs, bacterial methane oxidation was situated close to the subsequent succession stage, soils being ice-free for 10 yrs.

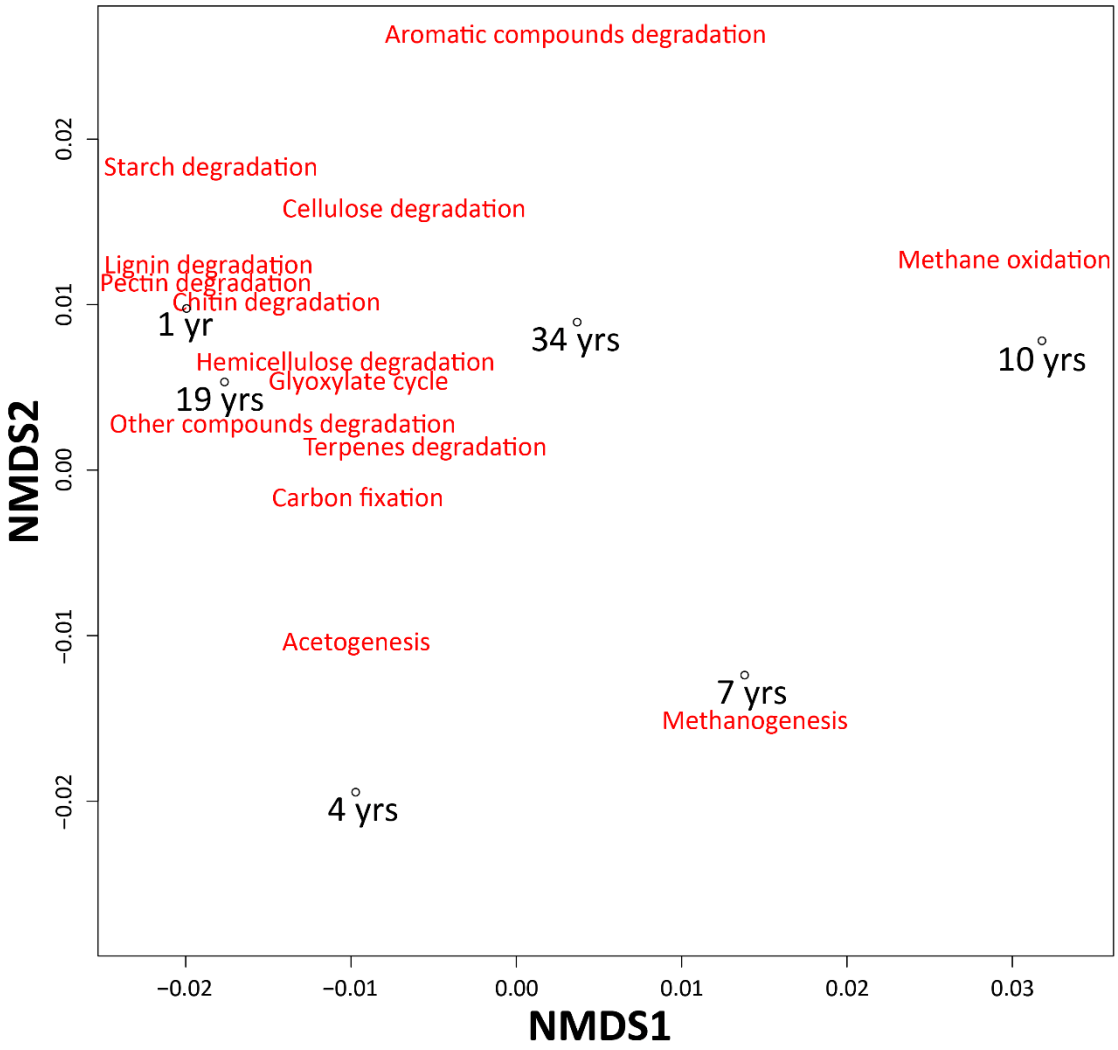
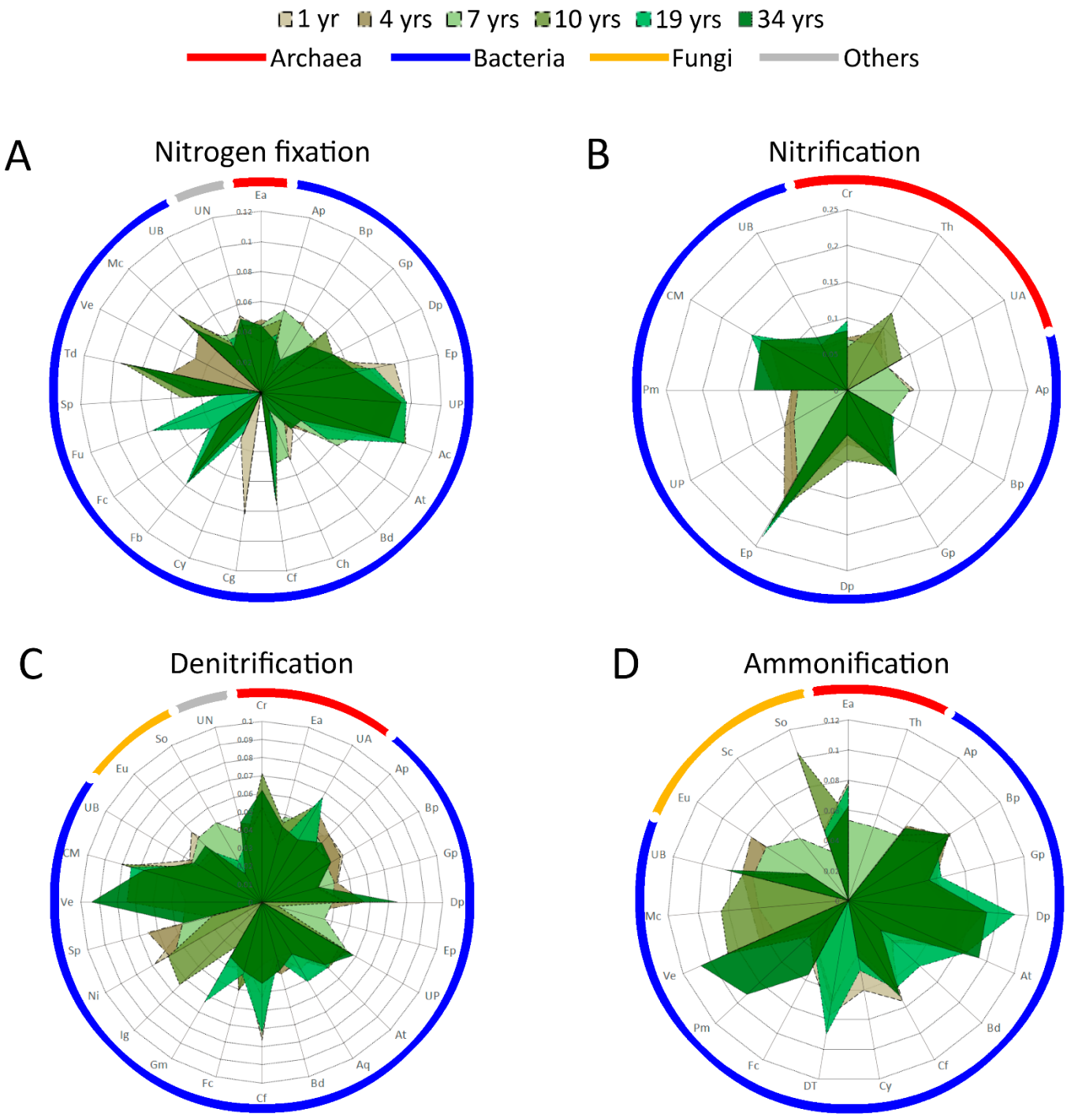


Fig. 2.5. Two dimensional non-metric multidimensional scaling (NMDS) ordination plots of sampling points (Bray-Curtis similarities) showing the distribution of the different C-cycling pathways along the glacier chronosequence. Succession stages are shown in black. Genes were grouped into different categories (in red).

Nitrogen metabolism

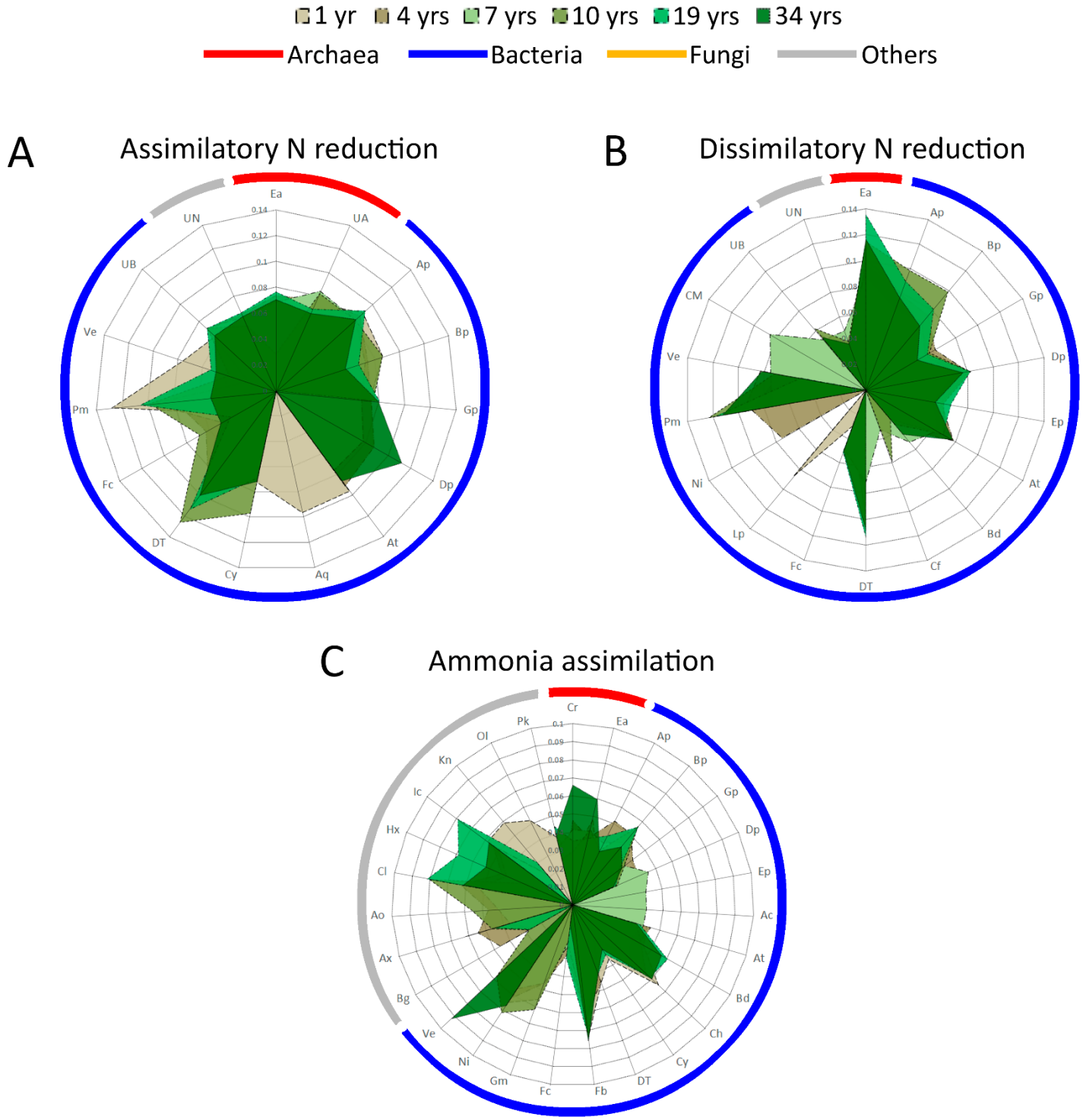
The potential for the major activities involved in N cycle (N_2 fixation, ammonification, ammonia assimilation, nitrification, assimilatory N reduction, dissimilatory nitrate reduction, denitrification and anammox) was detected for communities across all succession stages (Fig. 2.1). N fixation genes were detected among 23 taxa, with the highest signals *for Acidobacteria, Epsilonproteobacteria* and *Thermodesulfobacteria* from different succession stages (Fig. 2.1, 2.6a). The potential for nitrification was found in 2 archaeal and 8 bacterial taxa, with the highest signal for *Epsilonproteobacteria* (Fig. 2.6b). We found 24 taxa among archaeal, bacterial and fungal domains capable to remove soil nitrate via denitrification, with the highest signals for different taxonomic groups (*Verrucomicrobia, Deltaproteobacteria* and *Chloroflexi*) at different succession stages (Fig. 2.6c). The ability to ammonification was found in a total 18 taxa among archaea, bacteria and fungi with highest signals for bacterial taxa (Fig. 2.6d). Genes for assimilatory N reduction were found in *Euryarchaeota* and 12 bacterial taxa, showing the highest signals in *Planctomycetes*, *Deltaproteobacteria* and *Deinococcus-Thermus* (Fig. 2.7a). The potential for dissimilatory nitrate reduction was found in *Euryarchaeota* and 15 bacterial taxa, the highest signal were detected for *Euryarchaeota, Deinococus-Thermus* and *Planctonomycetes* genes (Fig. 2.7b). The ability to ammonia assimilation was found in a total 27 taxa among archaea, bacteria, fungi, algae and protozoa with highest signals for different taxonomic groups at different succession stages (Fig. 2.7c). The potential for annamox pathway was recognized only for *Planctomycetes* (Fig. 2.1) but with strong signal at every succession stage.

Nitrogen cycle pathways differed markedly along the chronosequence (Fig. 2.8). Most genes were associated to non-forest succession stages. N fixation gene (*nifH*) was plotted close to soils being ice-free for 1 and 4 yrs indicating that these potential are mainly associated to initial succession stage states. Denitrification genes were situated at the center of the ordination indicating that this activity is no clearly associated to a specific succession stage. The genes involved in nitrification were sited close to soils being ice-free for 1 yr, dissimilatory N reduction genes close to soils being ice-free for 7 yrs, asssimilatory N reduction genes close to soils being ice-free for 10 yrs and ammonia assimilation close to soils being ice-free for 19 yrs. Ammonification genes were ordinated near of soils being ice-free for 1 yr and 10 yrs.


1 yr
4 yrs
7 yrs
10 yrs
19 yrs
34 yrs
Archaea
Bacteria
Fungi
Others
A
Nitrogen fixation
B
Nitrification
C
Denitrification
D
Ammonification

◀ **Fig. 2.6.** Radar charts depicting taxa-function relationships for the nitrogen cycling genes. Relative signal intensity was normalized for the number of probes per taxon and expressed in percentage of total activity. Values from each sampling point were plotted for the nitrogen cycling genes involved in **(a)** N2 fixation; **(b)** Nitrification; **(c)** Denitrification and **(d)** Ammonification. Two-character codes denote microbial phyla: Archaea: Cr: *Crenarchaeota*, Ea: *Euryarchaeota*, Th: *Thaumarchaeota*, UA: Undetermined Archaea. Bacteria: Ap: *Alphaproteobacteria*, Bp: *Betaproteobacteria*, Gp: *Gammaproteobacteria*, Dp: *Deltaproteobacteria*, Ep: *Epsilonproteobacteria*, UP: Undetermined *Proteobacteria*, Ac: *Acidobacteria*, At: *Actinobacteria*, Aq: *Aquificae*, Bd: *Bacteroidetes*, Ch: *Chlorobi*, Cf: *Chloroflexi*, Cg: *Chrysiogenetes*, Cy: *Cyanobacteria*, DT: *Deinococcus-Thermus*, Fb: *Fibrobacteres*, Fc: *Firmicutes*, Fu: *Fusobacteria*, Gm: *Gemmatimonadetes*, Ig: *Ignavibacteriae*, Ni: *Nitrospirae*, Pm: *Planctomycetes*, Sp: *Spirochaetes*, Td: *Thermodesulfobacteria*, Ve: *Verrucomicrobia*, CM: *Candidatus Methylomirabilis*, Mc: *Magnetococcus*, UB: Undetermined Bacteria. Fungi: Do: *Dothideomycetes*, Eu: *Eurotiomycetes*, Sc: *Schizosaccharomycetes*, So: *Sordariomycetes*, Ag: *Agaricomycetes*. Others: Bg: *Bangiophyceae*, Ax: *Apicomplexa*, Ao: *Armophorea*, Cl: *Choanoflagellida*, Hx: *Hexamitidae*, Ic: *Ichthyosporea*, Kn: *Kinetoplastida*, Ol: *Oligohymenophorea*, Pk: *Perkinsida*. Un: Undetermined.

Fig. 2.7. Radar charts depicting taxa-function relationships for the nitrogen cycling genes. Relative signal intensity was normalized for the number of probes per taxon and expressed in percentage of total activity. Values from each sampling point were plotted for the nitrogen cycling genes involved in **(a)** Assimilatory N reduction; **(b)** Dissimilatory N reduction and **(c)** Ammonia assimilation. Two-character codes denote microbial phyla (see Figure 2.6). ▶


1 yr
4 yrs
7 yrs
10 yrs
19 yrs
34 yrs
Archaea
Bacteria
Fungi
Others
A
Assimilatory N reduction
Ea
UA
Ap
Bp
Gp
Dp
At
Aq
Cy
DT
Fc
Pm
Ve
UB
UN
B
Dissimilatory N reduction
Ea
Ap
Bp
Gp
Dp
Ep
At
Bd
Cf
DT
Fc
Lp
Ni
Pm
Ve
CM
UB
UN
C
Ammonia assimilation
Cr
Ea
Ap
Bp
Gp
Dp
Ep
Ac
At
Bd
Ch
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DT
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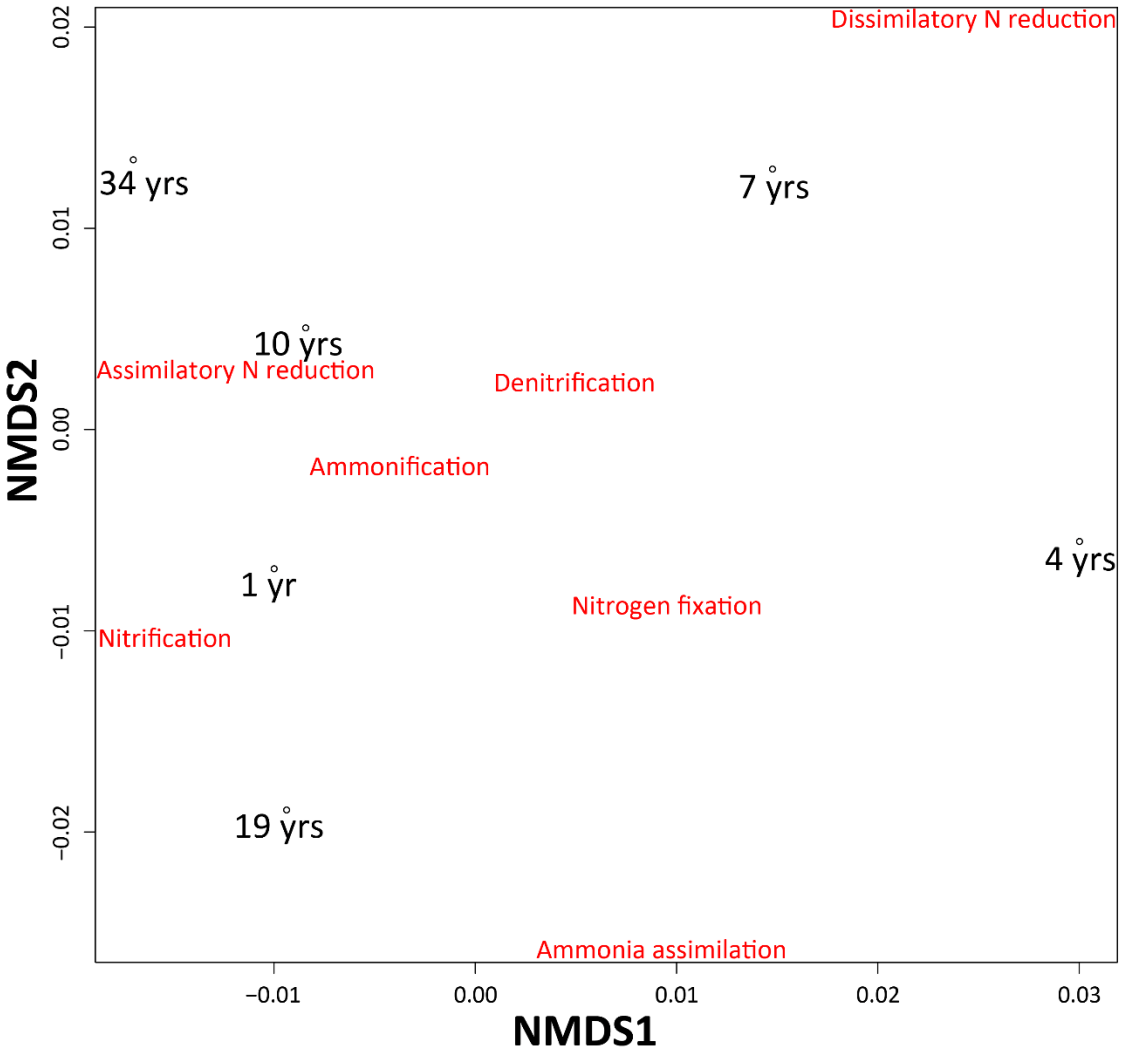


Fig. 2.8. Two dimensional non-metric multidimensional scaling (NMDS) ordination plots of sampling points (Bray-Curtis similarities) showing the distribution of the different N-cycling pathways along the glacier chronosequence. Succession stages are shown in black. Genes were grouped into different categories (in red).

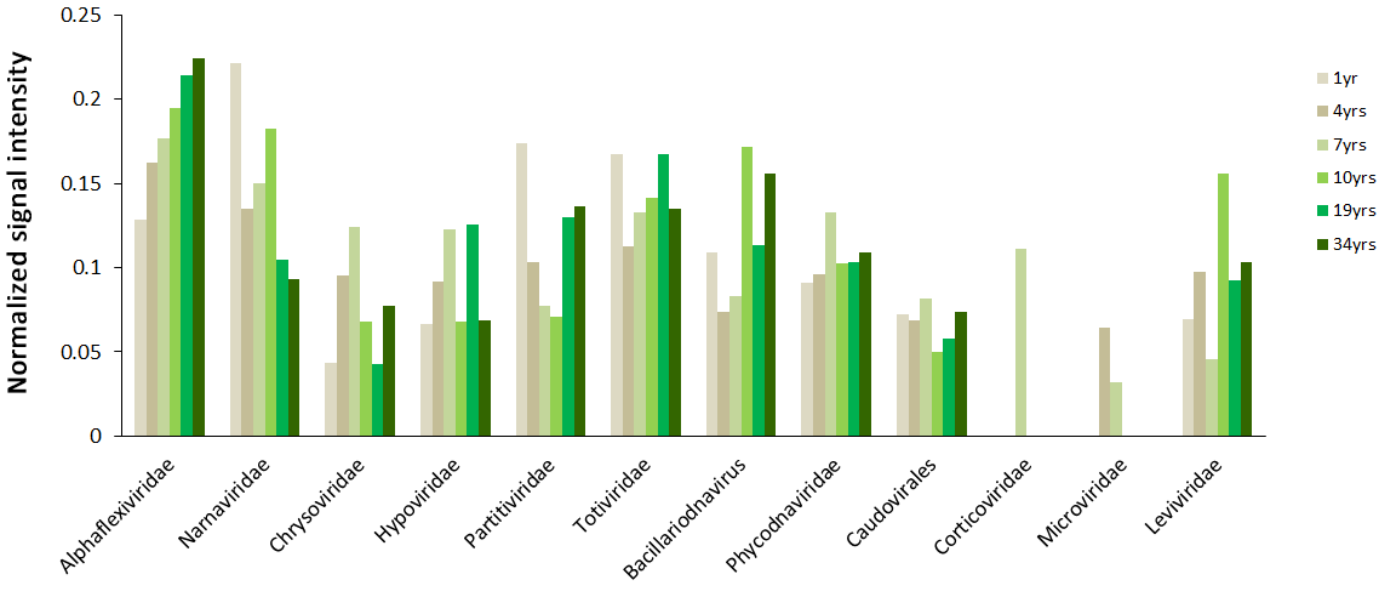
Stress responses

Archaeal, bacterial and fungal stress response genes to cold and heat shocks, desiccation, osmotic and oxidative stresses and glucose, oxygen, nitrogen and phosphate limitation were widespread along the chronosequence (Fig. 2.2). The highest signals were found for the genes less taxonomically widely distributed, such as genes for cold shock proteins found in a few groups of bacteria, for drought tolerance found in *Euryarchaeota* and some fungal groups, and for glucose limitation detected for

Euryarcheota and a few groups of bacteria. The signals were slightly more pronounced at soils that have been free of ice for 1 yr for the most of the stress response genes (Fig. 2.2).

Virus signatures and antibiotic resistance

Genes from a total of 12 viral taxa were detected along the chronosequence, eight corresponding to viruses with eukaryotic hosts and four infecting prokaryotes. The eukaryotic-infecting viral signatures included two families of ssRNA (*Alphaflexiviridae* and *Narnaviridae*) and four of dsRNA fungal viruses (*Chrysoviridae, Hypoviridae*, *Partitiviridae* and *Totiviridae*). One family of ssDNA (*Bacillariodnavirus*) and one of dsDNA algal viruses (*Phycodnaviridae*) were also detected. On its behalf, within the four bacteriophage families identified, two corresponded to dsDNA (*Caudovirales* and *Corticoviridae*), one to ssDNA (*Microviridae)* and one to ssRNA (*Leviviridae*) viruses. Every viral family was detected at every succession stage, except for two bacteriophage families, *Microviridae*, only present at soils being ice-free for 1 and 4 ysr, and *Corticoviridae*, only detected at soils being ice-free for 4 yrs (Fig. 2.9). *Alphaflexiviridae* (with fungi and plants as natural host) genes increase in abundance along the succession (Fig. 2.9). Additionally, micro-eukaryotic viruses showed relatively stronger signals than prokaryotic ones at every succession stage, especially at soils being ice-free for 10 and 19 yrs (Fig. 2.10).



◀**Fig. 2.9.** Relative abundance of viral families along the glacier chronosequence. Viral families were identified as 8 microeukaryotic-infecting viruses (6 fungal: *Alphaflexiviridae*, *Narnaviridae*, *Chrysoviridae*, *Hypoviridae*, *Partitiviridae* and *Totiviridae*; 2 algal viruses: *Bacillariodnavirus* and *Phycodnaviridae*) and 4 prokaryote-infecting viruses (*Caudovirales*, *Corticoviridae*, *Microviridae* and *Leviviridae*). Every viral family was detected at succession stage, with the exception of *Corticoviridae* and *Microviridae*.

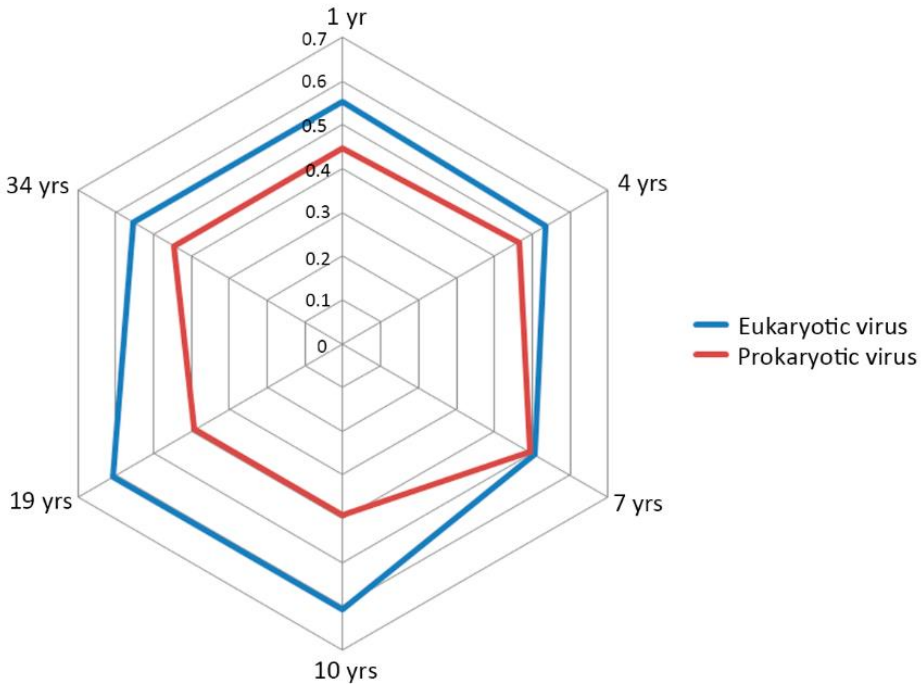


Fig. 2.10. Radar charts depicting relative signal intensity (normalized for the number of probes per taxon and expressed in percentage of total intensity) of viral groups inecting eukaryotic or prokaryotic hosts at each succession stage along Pia Glacier forefield chronosequence.

Diverse categories of fungal, bacterial and archaeal antibiotic resistance genes encoding for isopencillin, phenazine-1-carboxilic acid, bacitracin, erythromycin, lincomycin, p-aminobenzoic acid, 2,4-diacetylphloroglucinol, aminopyrrolnitrin, subtilin and streptomycin were detected at every sampling point (Table 2.3). The majority of them were of bacterial origin.

Table 2.3. Relative signal intensity of antibiotic resistance genes found in the GeoChip data. *pcb*C (isopencillin N synthase) and *phz*F (phenazine-1-carboxilic acid) were found in both eukaryotes and prokaryotes. Antibiotic resistance genes for prokaryotes were: *bac*A (bacitracin), *igr*D (erythromycin), *lmb*A (lincomycin), *pab*A (p-aminobenzoic acid), *phl*D (2,4-diacetylphloroglucinol), *phz*A (phenazine-1-carboxilic acid), *prn*D (aminopyrrolnitrin), *spa*R (subtilin) and *str*R (5'-hydroxystreptomycin).

		Gene	1 yr	4 yrs	7 yrs	10 yrs	19 yrs	34 yrs
Eukaryotic	Fungi	pcbC	0.046	0.040	0.044	0.049	0.051	0.055
		phzF	0.010	0.013	0.013	0.000	0.005	0.006
Prokaryotic	Archaea	phzF	0.005	0.004	0.000	0.000	0.000	0.000
	Bacteria	bacA	0.013	0.014	0.014	0.015	0.019	0.015
		igrD	0.022	0.020	0.022	0.023	0.022	0.026
		lmbA	0.018	0.015	0.016	0.019	0.018	0.018
		pabA	0.032	0.036	0.042	0.033	0.032	0.032
		pcbC	0.234	0.230	0.217	0.216	0.233	0.227
		phlD	0.012	0.009	0.020	0.019	0.012	0.013
		phzA	0.103	0.102	0.086	0.112	0.110	0.105
		phzF	0.463	0.480	0.490	0.476	0.457	0.458
		prnD	0.017	0.013	0.013	0.012	0.018	0.012
		spaR	0.011	0.012	0.006	0.012	0.011	0.006
		strR	0.013	0.014	0.017	0.014	0.013	0.026

2.4 Discussion

Soil microbial communities from Pia glacier forefield differed in their putative functionality along the studied chronosequence. Carbon and nitrogen fixation were associated primarily with initial succession stages (soils ice-free for 1 and 4 yrs). Our data indicates chemoautotrophic pathways also operate in the forefield soil, thus expanding the microbial capacity to incorporate C to these soils beyond photoautotrophy, a feature also observed in other soils in extreme environments (Freeman et al. 2009; Chan et al. 2013; Wei et al. 2015a). Pathways for nitrogen fixation were more commonly encountered for non-photosynthetic taxa. This highlights the importance of Proteobacteria and other taxa in nitrogen fixation, similar to observations made for Antarctic soils (Chan et al. 2013). Free-living *Cyanobacteria*, although abundant at certain succession stages, might have a lower contribution to the nitrogen fixation

process in soils than previously assumed (Crews et al. 2001; Yeager et al. 2004; Nemergut et al. 2007; Abed et al. 2010; Janatovká et al. 2013).

We observed a widespread distribution of genes for pathways of organic polymer degradation, although an interesting pattern emerged in that early successional stages were dominated by bacterial pathways whereas in later successional stages fungal pathways were more diverse. Organic C inputs could have occurred via glacial runoff, wind-blown detritus or mammal and bird droppings (Anesio and Laybourn-Parny 2012; Schulz et al. 2013; Zumsteg et al. 2002; Bradley et al. 2014), as well as ancient organic matter stored beneath the ice glacier (Bardgett et al. 2007). Our data suggest strong potential for microbial turnover of these carbon reservoirs, including C-1 pathways (methanogenesis and methane oxidation), and collectively they may be important in oligotrophic soils (Bardgett et al. 2007; Sattin et al. 2009; Brankatschk et al. 2011).

A strong phylum-specific role for microbial taxa in nitrogen cycling was indicated and occurrence was closely related to soil characteristics. Younger nitrogen-poor soils supported high levels of nitrogen fixing and ammonification pathways, but also evidence of pathways for net loss of nitrogen from the soil via denitrification. In soils ice-free for 1 and 4 yrs oxygen penetration could be facilitated by the lack of soils crust structure, which might favor the process of nitrification (Johnson et al. 2005; Brankatschk et al. 2013). Nitrification processes have not been previously associated to initial succession states of soil crusts development (Brankatschk et al. 2011), but the fast colonization rates observed in these soils exposed after the Pia glacier retreat (Fernández-Martínez et al. submitted) could facilitate a faster microbial colonization of N fixers and ammonifiers. The lack of nitrate plant assimilation processes can also contribute to the observed nitrate accumulation at soils being ice-free for 4 and 7 yrs. Dissimilatory N reduction (associated to soils being ice-free for 7 yrs), assimilatory N reduction (associated to soils being ice-free for 10 yrs) and denitrification (associated mainly to soils being ice-free for 7 and 10 yrs) could be facilitated by the accumulation of nitrates in previous succession stages. The ammonia assimilation which appeared associated to soils being ice-free for 19 yrs (attributed mainly to bacterial taxa) could be facilitated by the previous production of ammonia through bacterial assimilatory and dissimilatory N reduction.

The efficient use of available soil nutrients could be the main driver of the observed successional functional changes at glacier Pia forefield. At initial succession stages, microorganisms are mainly involved in anabolic and catabolic pathways that help to increase nutrient availability. The subsequent colonization by lichen, mosses and pioneer vascular plants is accompanied by more complex microbial transformations such as denitrification and methanogenesis. This phenomenon of nutrient microorganism-plant competence have been reported in different glacier forefields over the world (Hodge et al. 2000; Thébault et al. 2014; Cao et al. 2015; Zilla et al. 2015). In addition, the vegetation cover formed by lichen, mosses and pioneer herbs could facilitate the early colonization by taxonomic microbial groups with specific environmental requirements through their contribution to the formation of diverse microenvironments. Indeed for soils ice-free for 7 yrs where lichens, mosses and pioneer plants coexist, the proportion of endemic genes is higher, which suggests a higher diversity of microhabitats with favorable conditions for specific microbial activities. Anaerobic conditions could be favored by the water holding capacity of terricolous lichens (de los Ríos et al. 2011) and soil crusts (Belnap et al. 2001) thus facilitating the activity of methanogenic denitrifying bacteria and reducing nitrification processes (Johnson et al. 2005; Brankatschk et al. 2013) and inducing methanogenesis processes (Hofmann et al. 2013) in soils being ice-free for 7 yrs. The establishment of a consistent plant cover and the forest development and the consequent higher availability of nutrients in soils could result in dominance of catabolic microbial activities. In fact, organic soils from *Nothofagus* forest in the studied areas has been considered the major substrate for heterotrophic soil microbial communities (Thébault et al. 2014). The root exudation of carbohydrates or presence of plant litter in vegetated succession stages from the Pia forefield chronosequence can promote mobilization of both C and N from this organic matter because microbial communities have the potentials to degrade different organic compounds (Walton 1985; Yergeau et al. 2007; Bradley et al. 2014; Yue et al. 2015). Gene signatures encoding enzymes that can degrade complex organic substrates (starch, lignin, chitin and pectin) were associated primarily to pioneer communities of bacteria and fungi but they were also detected at succession stages with presence of *Nothofagus* trees and dominance of degradative fungal genes. Indeed, terpenes or hemicellulose degradation potentials were primarily associated to soils

being ice-free for 19 yrs, and the degradation of aromatic compounds (activities also involved in lignin degradation pathway) to soils being ice-free for 34 yrs. Metabolic activities involved in the degradation of complex organic compounds have been previously associated to highly vegetated soils from other glacier forefields located at the Northern Hemisphere (Bardgett et al. 2007; Hahn et al. 2013).

The lack of significant differences in stress response gene profiles along the studied chronosequence indicated that this is a generalized feature for microorganisms colonizing Pia glacier forefield. These results do not suggest the existence of a strong abiotic control at this level along the chronosequence. The slightly higher signal detected in microbial communities close to the glacier front could be related to the colonization by pioneer microorganisms with a wider range of stress tolerance strategies than colonizers of older soils (Sigler et al. 2002; Sigler and Zeyer 2004).

Distribution of the relevant frequency of viral signals along Pia Glacier forefield appeared intimately related to the presence of their potential hosts along the succession, suggesting that virus could play a role in potential control on microbial populations along this succession process. In fact, the viral activity could suppose a 'bottom-up' trophic regulatory mechanism on their hosts along the studied chronosequence, similarly to the mechanism described for Antarctic endolithic microbial communities (Wei et al. 2015b). This strategy would explain the higher proportion of microeukaryote-infecting virus at respect to prokaryote-infecting ones found in soils being ice-free for 10 and 19 yrs, where extensive plant colonization facilitates the colonization by mycorrhizal and saprophytic fungi (Fernández-Martínez et al. submitted). The restricted occurrence of the bacteriophage families *Microviridae* and *Corticoviridae* could be associated to the increase of soil water retention associated to lichen and moss colonization (de los Ríos et al. 2011; Raggio et al. 2012) due to these families have been described associated to specific hosts from moisture-sufficient soils (Wei et al. 2015b).

The identification of antibiotic resistance genes along Pia glacier forefield permit to suggest another potential biotic regulation of microbial community structure along primary succession, although marked successional patterns have not been found. The antibiotic resistance pathways could be involved not only in the defense against pathogens (Hibbing et al. 2009; Chan et al. 2013; Koskella and Breitbart 2014), but also

in rapid microbial competitive response induced by low availability of nutrients (Sigler and Zeyer 2004; Shank and Kolter 2009). This control could be characteristic of soil microbial communities because antibiotic resistance genes were not previously found in ice from Chile and Antarctica glaciers and attributed to the isolation of these areas (Segawa et al. 2013)

Successional replacement of putative metabolic pathways associated to changes in community structure has been evident along Pia glacier forefield succession. These results confirmed our expectations that functional community structure changes parallel to primary succession. We suggest nutrient availability is a key driver for microbial functionality in soils.

2.5 Supporting infomation

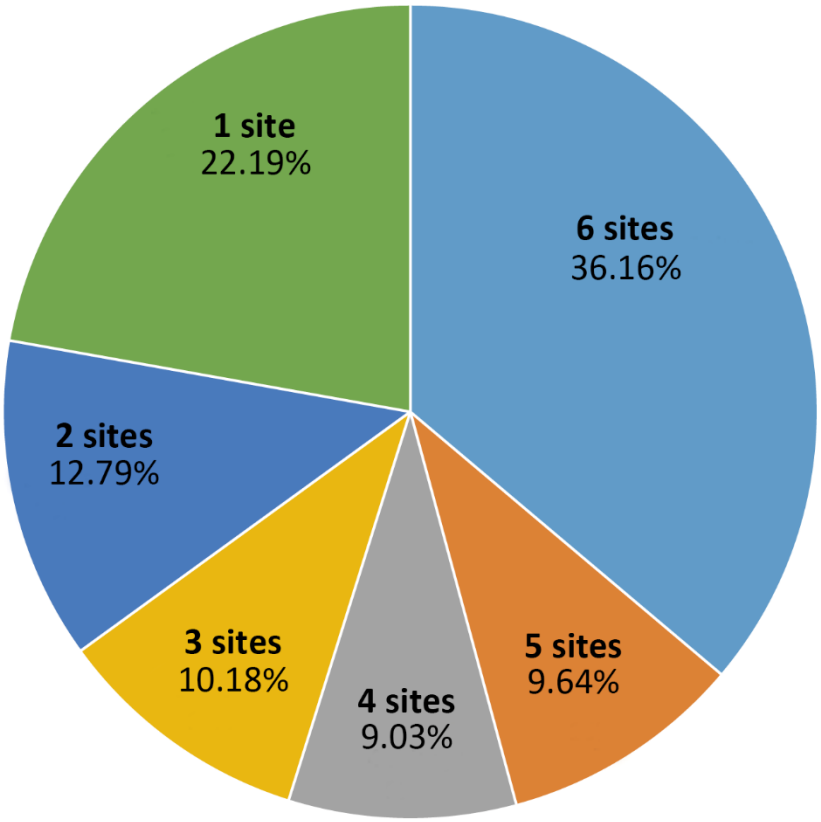
Supporting information Table 2.1. Major gene probes involved in Carbon cycling returning highest signal intensity.

Autotrphy	Calvin cycle	Ribulose-1,5-bisphosphate carboxylase/oxygenase	RuBisCO
		Carboxysome shell proteins	*cso*S1/*ccm*K
		Carbon dioxide-concentrating mechanism protein	*ccm*L
		Cytosolic fructose-1,5-bisphosphatase	FBPase
		Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
		Phosphoglycerate kinase	*pgk*
		Triosephospate isomarese	TIM
		Transketolase A	*tkt*A
	Acetyl-CoA metabolism	Propionyl-CoA/acetyl-CoA carboxylase	*pcc*
		Carbon-monoxide dehydrogenase	CODH
		ATP citrate lyase	*acl*B
	Glyoxylate cycle	Isocitrate lyase	*ace*A
		Malate synthase	*ace*B
Acetogenesis	Wood-Ljundahl/reductive Acetyl-CoA pathway	Formyltetrahydrofolate synthase	FTHFS
Methanogenesis	Final steps of methanogenesis	Heterosulfide reductase B subunit	*hdr*B
		Methyl coenzyme M reductase	*mcr*A
Methane oxidation	Oxidoreductase genes	Soluble methane monooxigenase	*mmo*X
		Particulate methane monooxigenase	*pmo*A
Carbon degradation	Starch degradation	Alpha-amylase	*amy*A
	Chitin degradation	Chitinase	*chi*
		Chitin deacetylase	*CDA*
		Acetylglucosaminidase	*nag*A
	Hemicellulose degradation	Arabinofuranosidase	*ara*
		Xylanase	*xyl*A
	Cellulose degradation	Cellobiase	*cba*
		Endoglucanase	*eng*
	Pectin degradation	Rhamnogalacturonan acetylesterase	RGAE
		Pectinase	*pem*
	Lignin degradation	Phenol oxidase	*PO*
		Glyoxal oxidase	*glx*
	Aromatic compounds degradation	Tannase	*tanLpl*
	Other compound degradation	Cutinase	*cut*
		Alphagalactosidase	*a/gla*
		Inulinase	*inu*
		Lactase	*lct*
		Metalloprotease	*MP*
		Phospholipase D	*PLD*

Supporting information Table 2.2. Major gene probes involved in Nitrogen cycling returning highest signal intensity.

N2 fixation	Nitrogenase reductase	*nif*H
Nitrification	Ammonia oxigenase	*amo*A
	Hydroxylamine oxidase	*hao*
Denitrification	Membranous nitrate reductase	*nar*G
	Cu-nitrite reductase	*nir*K
	Cytochrome-nitrite reductase	*nir*S
	Catalytic subunits of the nitric oxide reductase	*cnor*B/*nor*B
	Nitrous oxide reductase	*nos*Z
Ammonification	Urea amidohydrolase alpha subunit	*ure*C
Assimilatory Nitrate reduction	Assimilatory nitrate reductase	*nar*B
	Assimilatory nitrate reductase	*nas*A
	Periplasmatic nitrite reductase	*niR*
	Periplasmatic nitrite reductases	*nir*AB
Dissimilatory Nitrate reduction	Nitrate reductase	*nap*A
	Periplasmic c-type cytochrome nitrite reductase	*nrf*A
Ammonia assimilation	Glutamate dehydrogenase	*gdh*
ANAMMOX	Hydrazine synthase	*hzs*A
	Hydrazine oxidoreductase	*hzo*

Supporting information Fig. 2.1. Percentage of genes detected at one (endemic), two, three, four, five and six (ubiquitous) sampling points along the chronosequence. These groups account for 22.19%, 12.79%, 10.18%, 9.03%, 9.64% and 36.16% of total detected genes, respectively.



CAPÍTULO 3

Diversidad de los *Nostoc* endosimbiontes de *Gunnera magellanica* en Tierra del Fuego, Chile

Resumen

El calentamiento global está causando el retroceso del hielo en glaciares de todo el mundo, especialmente a lo largo de las últimas décadas en algunas áreas del planeta. Una de las más afectadas es la región de Tierra del Fuego (en el extremo sur de Sudamérica). La recolonización de las áreas recientemente deglaciadas de esta región por parte de plantas vasculares es iniciada por *Gunnera magellanica*, la cual forma asociaciones simbióticas con el género de cianobacterias *Nostoc*, característica que probablemente confiere ventajas en este proceso de colonización. Esta asociación simbiótica en el género *Gunnera* es destacable, ya que representa la única relación simbiótica conocida entre angiospermas y cianobacterias. El objetivo de este trabajo fue estudiar la diversidad genética de los *Nostoc* simbiontes en *Gunnera* a tres niveles jerárquicos diferentes: individuo, población y región. Se examinaron para ello tres regiones genómicas diferentes: un fragmento de la subunidad pequeña del gen del ARN ribosomal (*16S*), la subunidad grande del gen de RuBisCO unida con la secuencia de su promotor y una proteína similar a una chaperona (*rbc*LX), y la región del espaciador interno transcrito (ITS). La identidad de *Nostoc* como simbionte fue confirmada en el tejido infectado del rizoma que se analizó. Los filotipos aislados en el presente estudio estaban estrechamente relacionados con otros conocidas por formar simbiosis con otros organismos, tales como hongos formadores de líquenes o briófitos. Encontramos doce haplotipos únicos en el análisis de la región *16S* (subunidad pequeña) del ARNr, diecinueve haplotipos únicos en el análisis de la región ITS y cincuenta y siete en la región de las proteínas de RuBisCO (*rbc*LX). No se encontró variabilidad genética entre los *Nostoc* simbiontes dentro de una única planta hospedadora, mientras que las poblaciones de *Nostoc* entre diferentes plantas hospedadoras de un mismo lugar de muestreo revelaron diferencias importantes. Es importante destacar el hecho de que se encontró variabilidad interpoblacional entre suelos recién deglaciados y lugares que llevan más tiempo sin estar cubiertos por el hielo, así como entre lugares de muestreo situados más al este y los situados más al oeste y entre las vertientes norte y sur de la Cordillera Darwin. La estructura celular de la relación simbiótica fue observada mediante microscopía electrónica de barrido a bajas temperaturas (LTSEM), mostrando cambios en la morfología tanto de las células del cianobionte (con mayor número de células diferenciadas como heterocistes) como de las células de la planta (mostrando un

mayor tamaño). Las etapas de desarrollo de la simbiosis, incluyendo los diferentes estadios de las paredes celulares y de las membranas, así como de las matrices de exopolisacáridos (EPS), fueron también observadas.

Abstract

Global warming is causing ice-retreat in glaciers worldwide, most visibly over the last few decades in some areas of the planet. One of the most affected areas is the region of Tierra del Fuego (southern South America). Vascular plant recolonization of recently deglaciated areas in this region is initiated by *Gunnera magellanica*, which forms symbiotic associations with the cyanobacterial genus *Nostoc*, a trait that likely confers advantages in this colonization process. This symbiotic association in the genus *Gunnera* is notable as it represents the only known symbiotic relationship between angiosperms and cyanobacteria. The aim of this work was to study the genetic diversity of the *Nostoc* symbionts in *Gunnera* at three different, nested scale levels: specimen, population and region. Three different genomic regions were examined in the study: a fragment of the small subunit ribosomal RNA gene (*16S*), the RuBisCO large subunit gene coupled with its promoter sequence and a chaperon-like protein (*rbc*LX), and the ribosomal internal transcribed spacer (ITS) region. The identity of *Nostoc* as the symbiont was confirmed in all the infected rhizome tissue analysed. Strains isolated in the present study were closely related to strains known to form symbioses with other organisms, such as lichen-forming fungi or bryophytes. We found twelve unique haplotypes in the *16S* rRNA (small subunit) region analysis, nineteen unique haplotypes in the ITS region analysis and fifty-seven in the RuBisCO proteins region (*rbc*LX). No genetic variability was found among *Nostoc* symbionts within a single host plant, while *Nostoc* populations among different host plants within a given sampling site revealed major differences. Noteworthy, inter-population variation was also shown between recently deglaciated soils and more ancient ones, between eastern and western sites and between northern and southern slopes of Cordillera Darwin. The cell structure of the symbiotic relationship was observed with LTSEM microscopy, showing changes in morphology of both cyanobiont cells (differentiate more heterocysts) and plant cells (increased size). Developmental stages of the symbiosis, including cell walls and membranes and EPS matrix states, were also observed.

3.1 Introduction

Pioneer colonization by microorganisms is important for the later establishment of higher organisms on newly exposed substrata (Matthews 1992; Chapin et al. 1994; Hoppert et al. 2004; de los Ríos et al. 2011). The modification of soil conditions includes increased water retention, stabilization of micro- and macro- particles and incorporation of nutrients such as organic carbon by photosynthesis or nitrogen by nitrogen fixation (Liengen and Olsen 1997; Uliassi and Ruess 2002; Duc et al. 2009). These processes occur in a variety of environments, including recently deglaciated areas. Glacier retreat, visible nowadays in polar and subpolar areas of both hemispheres due to global warming (Rivera et al. 2000; Church and White 2006; Cazavane and Llovel 2010; Rahmstorf 2010), exposes to the atmosphere soils and rocks previously covered by ice. The first colonization events consist of psychrophilic microorganisms, including cyanobacteria (Wynn-Williams 1996; Vincent 2000), that may have been present in the crust under the ice (or within the ice itself Takeuchi 2011) or that reach the site just after exposure (Davey and Rothery 1993; Hodson et al. 2008). This is followed by colonization of exposed rocks and soil by other organisms such as lichens or bryophytes, and finally by the establishment of vascular plants (Grubb 1986; Frenot et al. 1995, 1998; Hoppert et al. 2004; de los Ríos et al. 2011). In this process, symbioses (such as lichens, mycorrhizae or plant-bacteria symbiotic associations) play an important role, enabling or increasing the colonization abilities of the organisms involved, mainly by means of nutrient acquisition (Walker 1993). There is a large range of plants and microorganisms able to establish symbiotic relationships (reviewed by Stewart 1980; Stewart 1983; Smith and Douglas 1987; Meeks 1998; Bever et al. 2010), including plants such as legumes or cycads (Ow 1999; Costa et al. 2004; Tajhuddin et al. 2010) and microbes such as *Proteobacteria* (*e.g. Rhizobium*) and filamentous, heterocyst-forming cyanobacteria (genera *Nostoc* and *Anabaena*, Rasmussen et al. 1996). Of particular interest is the symbiotic relationship between the plant genus *Gunnera* (*Gunneraceae*) and its cyanobacterial endosymbiont, *Nostoc* spp., as it is the only documented specific association between angiosperms and cyanobacteria (Bergman et al. 1992; Söderbäck and Bergman 1993; Rasmussen et al. 1996; Black and Osborne 2004; Osborne and Bergman 2009). The genus *Gunnera* consists of 61 species distributed mainly in the Southern Hemisphere, with only two species present in Tierra del Fuego (Wilkinson and

Wanntorp 2007). These plants are perennial herbs, ranging in size from 30 cm (*G. magellanica*) to more than 5 m (Wilkinson and Wanntorp 2007). On the other hand, the genus *Nostoc* (*Nostocaceae*) comprises filamentous photosynthetic cyanobacteria that can form motile filaments (called hormogonia) and differentiate into atmospheric nitrogen-fixing cells (called heterocysts); these organisms can occur in a symbiotic state or free-living, in both terrestrial and aquatic habitats, and show a cosmopolitan distribution (Dodds et al. 1995). The intracellular symbiosis of *Nostoc* within the host *Gunnera* and the process of infection have been extensively studied (Bergman et al. 1992; Söderbäck and Bergman 1993; Rasmussen et al. 1996). When the symbiosis is established, many of the vegetative *Nostoc* cells become heterocysts that fix and transfer nitrogen to the host cell (Bergman et al. 1992). The host plant in turn exports carbon to the cyanobacterial symbiont and allows the cyanobacteria to expand their ecological niche. Multiple *Nostoc* strains are known to be able to establish symbiotic associations with different *Gunnera* species (Bergman et al. 1992; Rasmussen and Svenning 2001; Guevara et al. 2002). However, it has been addressed that a single plant might be associated only with a single *Nostoc* strain (Guevara et al. 2002) as a consequence of the timing and the mechanisms of the infection process.

The aims of the present study were to investigate the genetic diversity of symbiotic *Nostoc* strains associated with *Gunnera magellanica* at different spatial scales: within the same individual host plant and within and among several populations along Tierra del Fuego (including retreated glaciers and nearby areas with well-established ecosystem). Finally, we also aimed to characterize the relationship between the symbionts.

3.2 Materials and Methods

Study area

Gunnera magellanica specimens were collected in nine localities along Tierra del Fuego (XII Region, Chile). Six localities were sampled in Isla Grande, corresponding to areas where the glacial ice has been retreating over a period of time (Holmlund and Fuenzalida 1995). One locality on Península Brunswick and two on Isla Navarino were also sampled (Table 3.1).

Table 3.1. Collecting data for each of the localities studied

Site	Latitude	Longitude	No. of specimens	Collector	Date of collection
Site 1: Isla Grande de Tierra del Fuego. Bahía Ainsworth	S 54°29'10.1"	W 69°36'52.4"	12	S. Pérez-Ortega	December 2009
Site 2: Isla Grande de Tierra del Fuego. Bahía Fitton	S 54°25'55"	W 70°7'48"	15	S. Pérez-Ortega	December 2009
Site 3: Isla Grande de Tierra del Fuego. Seno Agostini	S 54°24'12"	W 70°26'24"	13	S. Pérez-Ortega	December 2009
Site 4: Isla Grande de Tierra del Fuego. Glaciar Pía 1	S 54°42'36"	W 69°42'1"	14	S. Pérez-Ortega	December 2009
Site 5: Península Brunswick	S 53°50'46"	W 71°7'0"	13	S. Pérez-Ortega	December 2009
Site 6: Isla Grande de Tierra del Fuego. Bahía Parry	S 54°46'43"	W 69°42'1"	12	S. Pérez-Ortega	December 2009
Site 7: Isla Grande de Tierra del Fuego. Glaciar Pía 2	S 54°34'11.6"	W 69°8' 6.32"	10	S. Pérez-Ortega	December 2009
Site 8: Isla Navarino. Nothofagus forest	S 54°56'55.97"	W 67°38'23.40"	14	M. Arróniz - Crespo	January 2011
Site 9: Isla Navarino. Cerro Bandera tundra	S 54°57'57.69"	W 67°38'21.79"	10 (7 for rbcLX)	M. Arróniz - Crespo	January 2011

The area is dominated by a rugged landscape, with Cordillera Darwin as the most important mountain range (reaching altitudes of 2,580 m.a.s.l.); high-altitude plateaus are also present. These sites have a maritime climate along the coast, characterized by hurricane-force winds and dense cloud cover (Burgos 1985). These features, combined with the influence of the Pacific Ocean and the peculiar, distinctive orography create a significant gradient of decreasing rainfall from the Atlantic towards the Pacific shore (500mm per year max near easternmost *Site 8* and *Site 9 versus* 4,000mm near westernmost *Site 5*) and between north and south slope of Cordillera Darwin (Koppes et al. 2009). The average temperature is 5° C with little seasonal changes near the seaside (Molina 1983; Xercavins Comas 1984; Endlicher and Santana Aguila 1988; Koremblit and Forte Lay 1991).

Maps and geographic representation were carried out using the software DIVA-GIS and the information from its webpage (Hijmans et al. 2001).

Biological material

Gunnera magellanica (*Gunneraceae*) is an herbaceous plant species present only in the southernmost region of the Southern Hemisphere. It is a perennial diploid (2n=34) plant that can be monoecious or dioecious, being much smaller in size compared to other species of the genus, which is mainly tropical. Diminutive size may represent an adaptation to more challenging environments (Wilkinson and Wanntorp 2007). Cyanobacteria-infected regions (n=181) inside the rhizome of complete *Gunnera magellanica* specimens (n= 133) were collected and stored individually at -20° C (samples from *Sites 1-7*), while the samples from *Site 8* and *Site 9* were immersed in CTAB buffer (Cetyl Trimethyl Ammonium Bromide, Cubero et al. 1999), and transported to the laboratory.

From each plant, at least one symbiotic colony was extracted in aseptic conditions, avoiding as much as possible the inclusion of surrounding vegetal tissue, as well as fragments of non-cyanobacteria infected rhizome tissue from four different specimens used as negative controls. The DNA extraction was carried out according to Cubero et al. (1999), with a modification at the lysis step, extending it to a 12-hour length.

PCR amplification and sequencing

Three different regions from the cyanobacterial genome were amplified: a fragment of the 16S ribosomal RNA gene (hereafter *16S*); the 16S-23S internal transcribed spacer (hereafter ITS); and the RuBisCO large subunit gene coupled with its promoter sequence and chaperon-like proteins (hereafter *rbc*LX). Reaction mix was carried out following O'Brien et al. (2005ab), completing a 25 µl-final volume, consisting of: dNTPs (0.2mM of each), 1.5mM of $MgCl_2$, 0.625 units of TaqPolimerase (1 unit·μl^{-1} BioTools, Madrid, Spain), 25 µg of BSA, 0.5 µM primers (forward and reverse) and 1x PCR buffer. The cyanobacterial specific primer pair CX-CW (Rudi et al. 1998) was used for the amplification of the *rbc*LX, using the following thermocycle conditions: a first 4-minute step at 94° C followed by 36 cycles of three steps: 94° C for 30 s, 55° C for 30 s, 72° C for 2 min and a final step of 72° C for 7 min. The *16S* and ITS sequences were amplified jointly using the primer pair 359F (Nübel et al. 1997) and 373R (Wilmotte et al. 1993), following a 'Touchdown PCR' protocol, whose conditions followed Janse et al.

(2003): an initial step at 94° C for 5 min followed by 20 cycles of three steps: 94° C for 1 min, 62-52° C for 1 minute (descending 0.5° C per cycle, the first being performed at 62° C and the last at 52° C) and 72° C for 1.5 min; 10 cycles of other three steps (94° C for 1 min, 52° C for 1 min and 72° C for 1.5 min) and a final step of 72° C for 30 min. All PCR amplifications were carried out in either a MJ Mini Personal Thermal Cycler (BIO-RAD) or a GeneAmp PCR System 2400 (Applied Biosystems). PCR products were purified using the UltraClean PCR Clean-Up kit (MoBio Laboratories INC.). Both DNA directions (5'-3'/3'-5') were sequenced, with the same primer pairs used in the amplification step, by Macrogen INC. laboratories (South Korea) through its automatic standard sequencing service, using a 3730XL DNA sequencer under required conditions by BigDye™ terminator cycle sequencing kit. DNA extracted from non-cyanobacteria infected plant rhizome tissue could not be amplified with the primer pairs used.

Sequence alignment, genetic diversity and phylogenetic analyses

Complementary sequences from the same specimen and DNA region were collapsed into contigs using SeqMan software (Lasergene v. 7.00, DNASTAR). Approximate identifications were obtained comparing contigs against the GenBank database by means of the BLAST algorithm (Thompson et al. 1994) in order to check for contaminations, using 97% of sequence coverage and E-value of 0.001 parameters as threshold in the searches. Alignments were made using the software ClustalW (Hall 1999), implemented within BioEdit v. 7.0.9 (Huber et al. 2004), being carried out for each of the three genomic regions. Furthermore, *rbc*LX sequences obtained from the *Gunnera* specimens were aligned with sequences from *Gunnera* chloroplasts retrieved from GenBank database in order to discard previously undetected plant organelle contamination. Bellerophon (Atschul et al. 1990) software was used to look for possible chimeras in all the alignments. Datasets with ambiguously aligned regions (*rbc*LX gene) were treated with the software Gblocks v. 0.91b (Castresana 2000) prior to phylogenetic analyses.

Alignments were collapsed into haplotypes with the software Collapse 1.2 (David Posada, available at http://darwin.uvigo.es/software/collapse.html). Genetic diversity measurements were computed in DnaSP v.5 (Librado and Rozas 2009). The following parameters were calculated: number of polymorphic or segregating sites, *S*; total

number of mutations, *Eta*; haplotype diversity, *Hd* (Nei 1987); and nucleotide diversity, π (Nei 1987). Statistical parsimony (Templeton et al. 1992) was used for later generation of genealogies (haplotype networks) using TCS v. 1.21 software (Clement et al. 2000). MOTHUR v. 1.21.1 (Schloss et al. 2009) was employed in order to cluster the sequences into OTUs (Operational Taxonomic Unit) for posterior phylogenetic analyses and for study of their relative abundance by the furthest neighbor method. Mantel tests were carried out by using the Microsoft Excel software complement GenAlEx (Smouse et al. 2008) to test the role of geographic distance in the genetic structure of the endosymbionts. The genetic structure of the populations of *Nostoc* associated with *G. magellanica* was checked using the analysis of molecular variance, AMOVA, (Excoffier et al. 1992) for *16S* and *rbc*LX genes using the software Arlequin v. 3.11 (Excoffier et al. 2005). In these analyses, different groups were structured, in order to find out which factors –environmental components of the sites, approximate time of exposition after ice retreat (Holmlund and Fuenzalida 1995; Koppes et al. 2009) or position concerning Cordillera Darwin, *i.e.* North *vs* South slope– better explain the genetic variance in the dataset.

Phylogenetic analyses were carried out for the *16S* and *rbc*LX genes. Collapsed haplotypes were aligned by using of ClustalW (Hall 1999) with the most similar sequences found in BLAST searches (Thompson et al. 1994) in the GenBank database. Most likely nucleotidic substitution models for the alignments were searched by means of the software jModelTest (Posada 2008) using the Akaike information criterium (Akaike 1974). The GTR (General Time Reversible, Tavaré 1986) +I +G was chosen for the *16S* and the *rbc*LX alignments. Maximum Likelihood phylogenetic analyses (Hasegawa et al. 1991) were then performed, estimating support for each node using bootstrapping (10000 repetitions), by the Nearest Neighbor Interchange method (NNI) implemented in the software MEGA 5.05 (Tamura et al. 2011). Phylogenetic analyses based on Bayesian inference (Yang and Rannala 1997) were carried out with Mr. Bayes software (Ronquist and Huelsenbeck 2003), performing 30 mill generations for *16S* and 17 mill for *rbc*LX.

Low Temperature Scanning Electron Microscopy (LTSEM)

Small pieces of vegetal tissue hosting cyanobacterial colonies were observed by LTSEM method by being mechanically fixed onto the specimen holder of a cryotransfer system (Oxford CT1500), plunged into subcooled liquid nitrogen, and then transferred to the microscope's preparation unit via an air-lock transfer device following the protocol described by de los Ríos et al. (1999). The frozen plant tissue was cryo-fractured in the preparation unit and transferred directly via a second air lock to the microscope cold stage, where it was etched for 2 min at −90° C. After ice sublimation, the etched surfaces were sputter-coated with gold in the preparation unit and the tissue then placed on the cold stage of the SEM chamber. Fractured surfaces were observed under a DSM 960 Zeiss SEM microscope at −135° C.

3.3 Results

Fifteen plants from different sampling sites were used for the intra-specimen diversity study. 63 sequences for each of the three genomic regions were obtained by the amplification of DNA extracted from the colonies (with at least three sequences from different colonies per plant) for the intra-specimen diversity study. The sequences obtained from different colonies within the same host specimen were identical. Further, analyses of tissue from 133 plants generated 113 sequences each for the *16S* and ITS regions and 110 for the *rbc*LX from those same specimens of *Gunnera magellanica* that were subsequently used for the population study level. All sequences were identified as belonging to *Nostoc* through BLAST searches in GenBank. Moreover, no chimeras were found in any alignment by the Bellerophon software analysis

Polymorphism analyses for each genomic region and for each sampling site showed different ranges of genetic diversity (Table 3.2). All polymorphic parameters for *16S* showed their lowest values in *Site 1* and *Site 9*. ITS region presented its lowest values for every parameter in *Site 1*. However, *rbc*LX gene region reached its lowest values for most of the polymorphic parameters in *Site 2* and *Site 9*, except for haplotype diversity, which was reached in *Site 1*. In *16S* gene analyses, highest levels for each parameter were reached in *Site 3*. Parameters for ITS chromosomal region reached these maximum

values in *Site 2* and for *rbc*LX gene in *Site 3*, but for haplotype diversity, which reached its maximum value in *Site 5* for both regions.

Table 3.2 Analysis of symbiotic *Nostoc* sequences diversity based on three genomic regions.

Parameters	Genomic regions		
	16S	ITS	*rbc*LX
General results			
No. of sequences	113	13	110
Sequece length (no. of nucleotides)	1040	88	907 (630)
Total no. of analysed sites	1040	80	619 (461)
Max. no. of total haplotypes	12	19	57 (32)
No. of polymorphic sites (*S*)	41 (sites 1-9/site 3)	26 (site 1/site 2)	115 (81; sites 2-9/site 3)
Total no of mutations (Eta)	45 (sites 1-9/site 3)	33 (site 1/site 2)	152 (85; sites 2-9/site 3)
Haplotypes			
Haplotype diversity (Hd)	0.787±0.02 (sites 1-9/site 3)	0.841±0.017 (site 1/site 5)	0.920±0.017 (0.867±0.018; site 1/site 5)
Nucleotides			
Diversity (π)	0.01606 (sites 1-9/site 3)	0.05783 (site 1/site 2)	0.06922 (0.04753; site 2-9/site 3)
Average no. of nucleotide differences (*k*)	16.702 (sites 1-9/site 3)	4.627 (site 1/site 2)	31.978 (21.910;sites 2-9/site 3)

For *rbcLX*, after-Gblocks results appear between parentheses. Sampling sites with the lowest and the highest values appear between brackets for all genomic regions.

Haplotypes found in each site and their relative abundance for *16S*, ITS and *rbc*LX regions are shown in Figs. 3.1, 3.2 and 3.3, respectively. For the *16S* gene, the lowest diversity was found in *Site 1* and *Site 9* with only one haplotype, followed by *Site 6* and *Site 7* with two. The greatest diversity was found in *Site 5* (five haplotypes). Except for *Site 7* and *Site 3*, the rest of sites showed a predominant haplotype (Fig. 3.1). Most of the haplotypes seem to be fixed; eight of them occurred in only at a single locality. On the other hand, haplotype 12 (present in two sites) and haplotypes 1, 2 and 5 (present in four sites) showed a broader distribution.

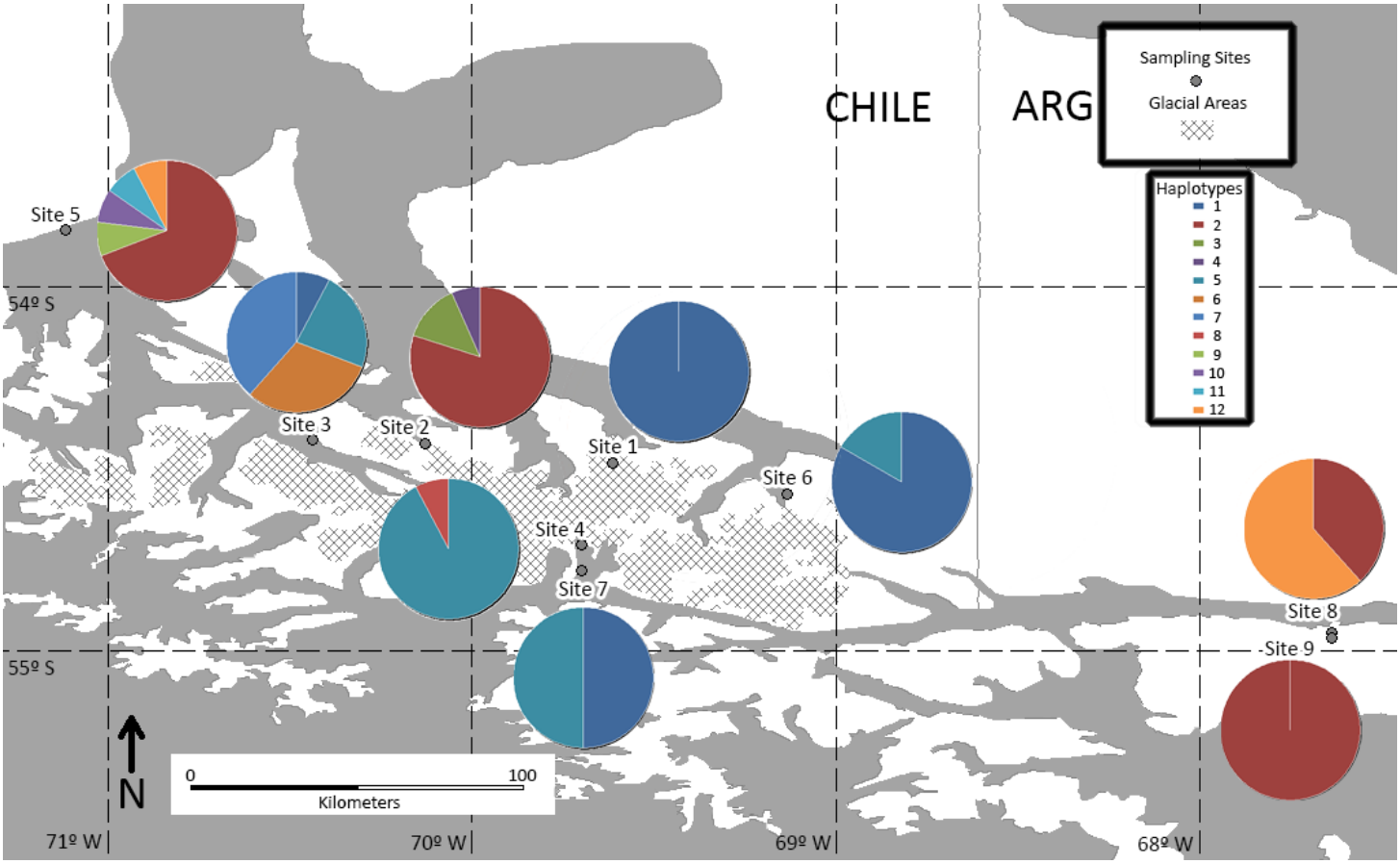


Fig. 3.1. Map of sampling area showing haplotypic diversity in different sites and relative abundance of each haplotype for *16S* gene data.

For the ITS region, only *Site 1* showed one unique haplotype, whereas the rest showed at least two (Fig. 3.2). *Site 5* showed the highest diversity with eight haplotypes. All sampling sites showed a predominant haplotype except for *Site 7* and *Site 3*, represented by two equally abundant haplotypes. The broadest distribution corresponded to haplotypes number 1 and 6 (occurring in four sites), followed by 2 and 14 (three sites) and 4, 10 and 18 (two sites).

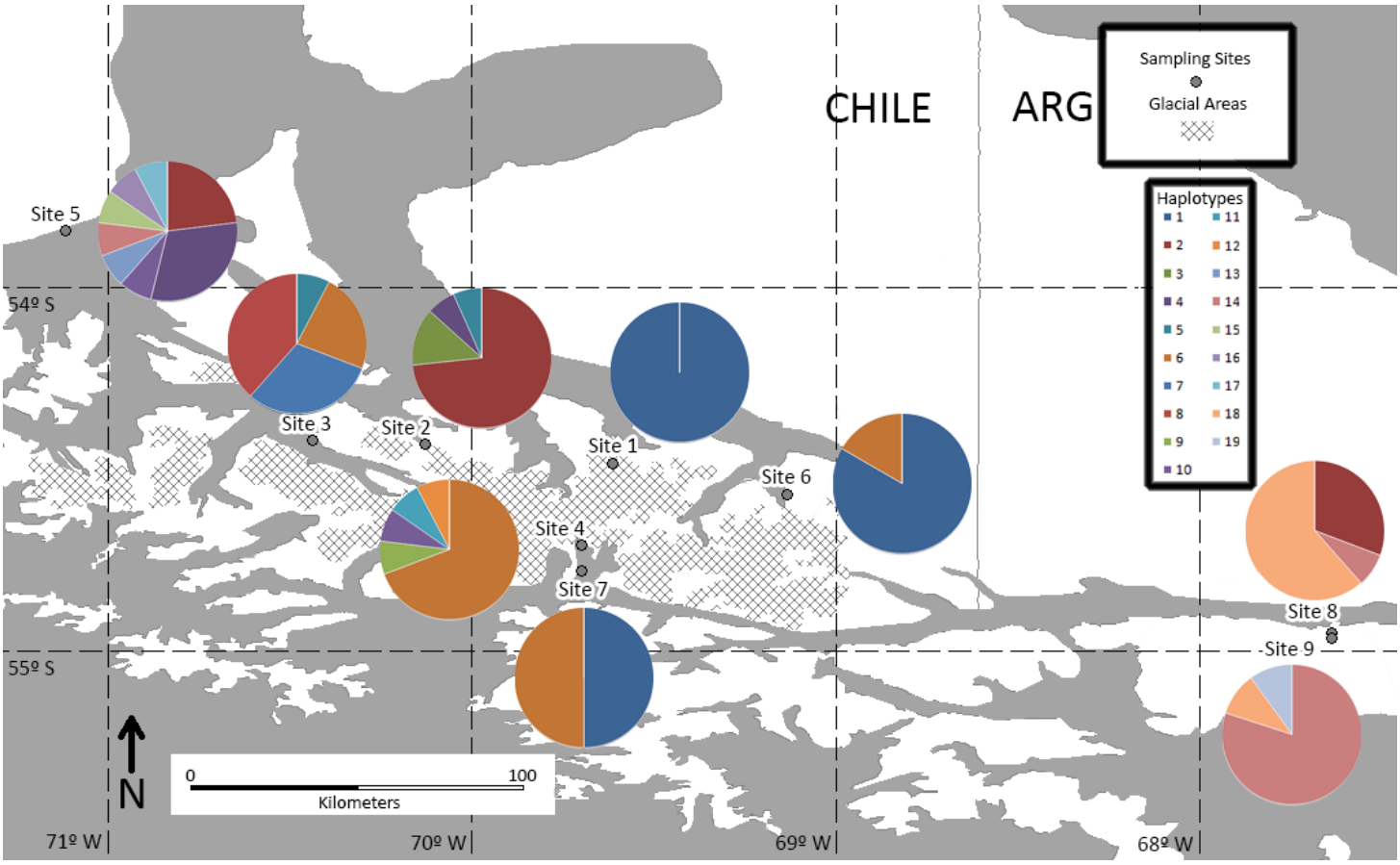


Fig. 3.2. Map of sampling area showing haplotypic diversity in different sites and relative abundance of each haplotype for ITS genomic region data.

Regarding the *rbc*LX region, all sites showed more than one haplotype (Fig. 3.3). *Site 9* showed the lowest number of haplotypes (two), while *Site 5* showed the highest genetic diversity with twelve haplotypes. Every site had a haplotype with more sequences than the others within it, except for *Site 1* and *Site 6*, which showed two and three respectively with the same number. Haplotypes number 2 and 11 showed the broadest distribution, being present in four sites; haplotype 31 occurred in three sites, and the rest occurred only in one site.

Fig. 3.4 shows how unique haplotypes are related by statistical parsimony, and their distribution among sampling sites, for the sequences of genomic *16S* gene (a) and ITS (b) regions. Two unconnected groups under a 95% parsimony criterion (Templeton et al. 1992) were found in the haplotypes of the 16S ribosomal gene (Fig. 3.4a). The first includes 59 sequences in four haplotypes (I, II, III, IV), while the second includes the other 44 sequences in 8 haplotypes (V, VI, VII, VIII, IX, X, XI, XII).

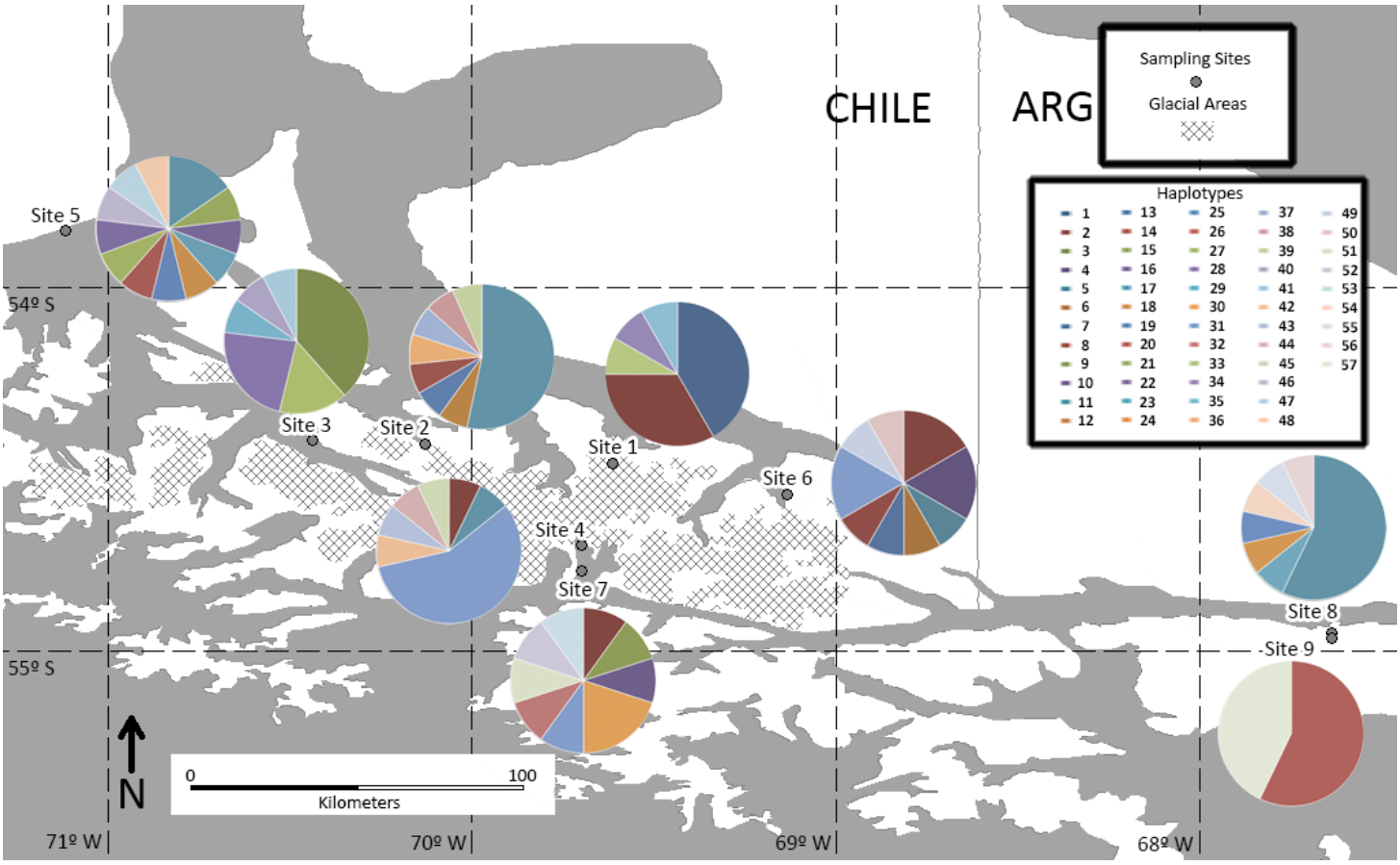
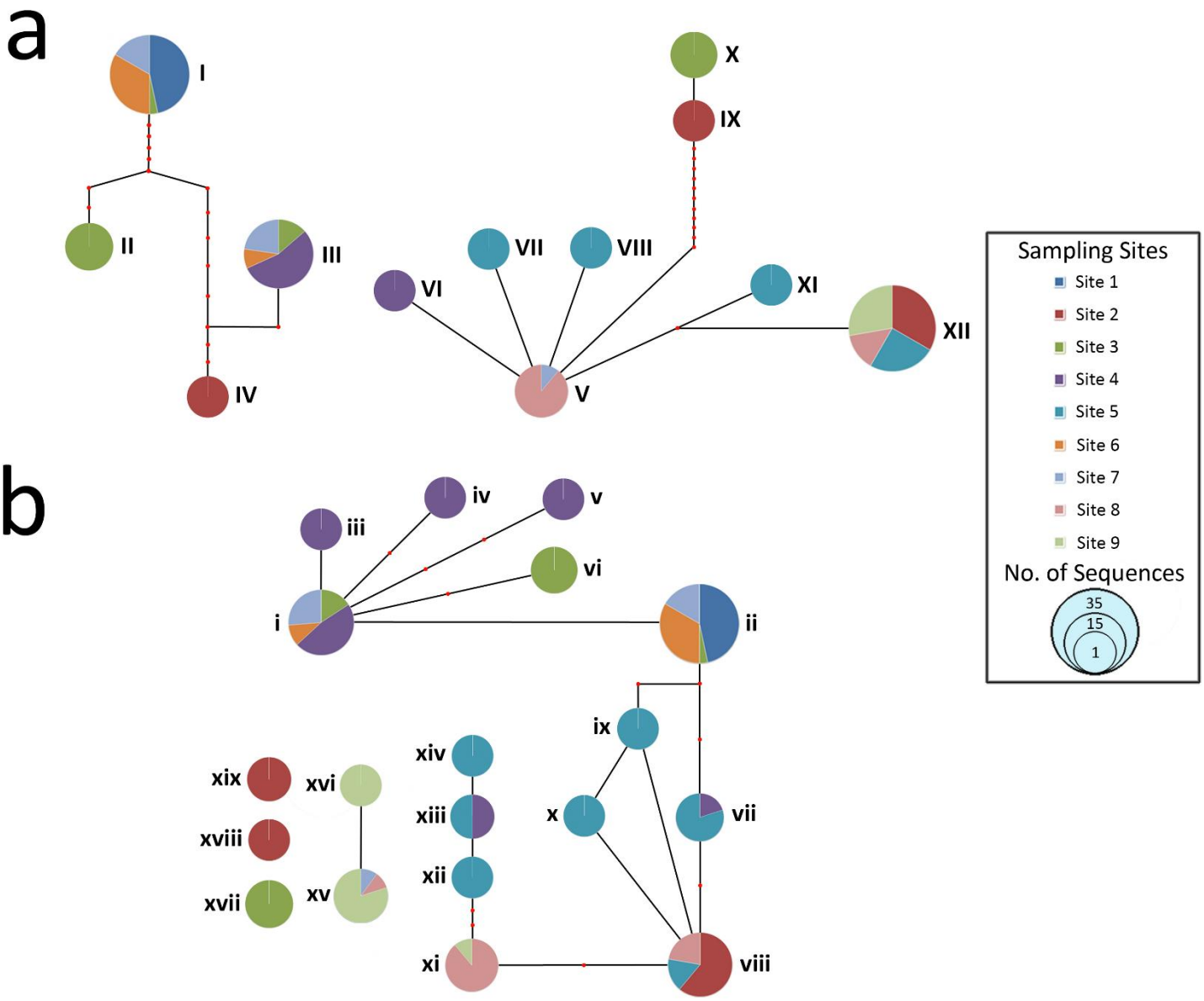


Fig. 3.3. Map of sampling area showing haplotypic diversity in different sites and relative abundance of each haplotype for *rbc*LX gene data.

On the other hand, haplotypes from the ITS region were included in five different groups in the network analysis (Fig. 3.4b). Three of them (xvii, xviii, xix, with 5, 1 and 2 sequences, respectively) were shown to be completely separated from the rest; two (xv, xvi, with 11 sequences) were only related to each other, while the fourteen remaining haplotypes (67 sequences) showed connection under this probability criterion.

Fig. 3.4. Statistical parsimony networks depicting relationships among analyzed haplotypes. Each circle represents one haplotype and its surface area is directly related with the number of sequences integrated in it. Roman numerals refer to posterior comments. Red dots along branch lines represent the number of nucleotide substitutions between two haplotypes. **(a)** Haplotypes found in the sequences of *16S* gene dataset; **(b)** Haplotypes found in the sequences of ITS region dataset. ▶



For the *16S* gene, at the level of 3% dissimilarity (indicating putatively distinct species) 2 OTUs were found, while at 5% dissimilarity (indicating congeneric taxa) a single grouping was observed (Table 3.3). The ITS region showed 12 OTUs while the *rbc*LX gene presented 23 OTUs (6 after Gblocks trimming), using 3% sequence dissimilarity. For the 5% cut-off value, the number of OTUs was reduced to 8 for ITS and to 15 (4 after trimming) for *rbc*LX. Only at 20% dissimilarity for ITS and at 18% (8% if Gblocks trimming is applied) for the *rbc*LX gene could 2 OTUs be recognized. These two OTUs included the same specimens independently of the analyzed genomic region.

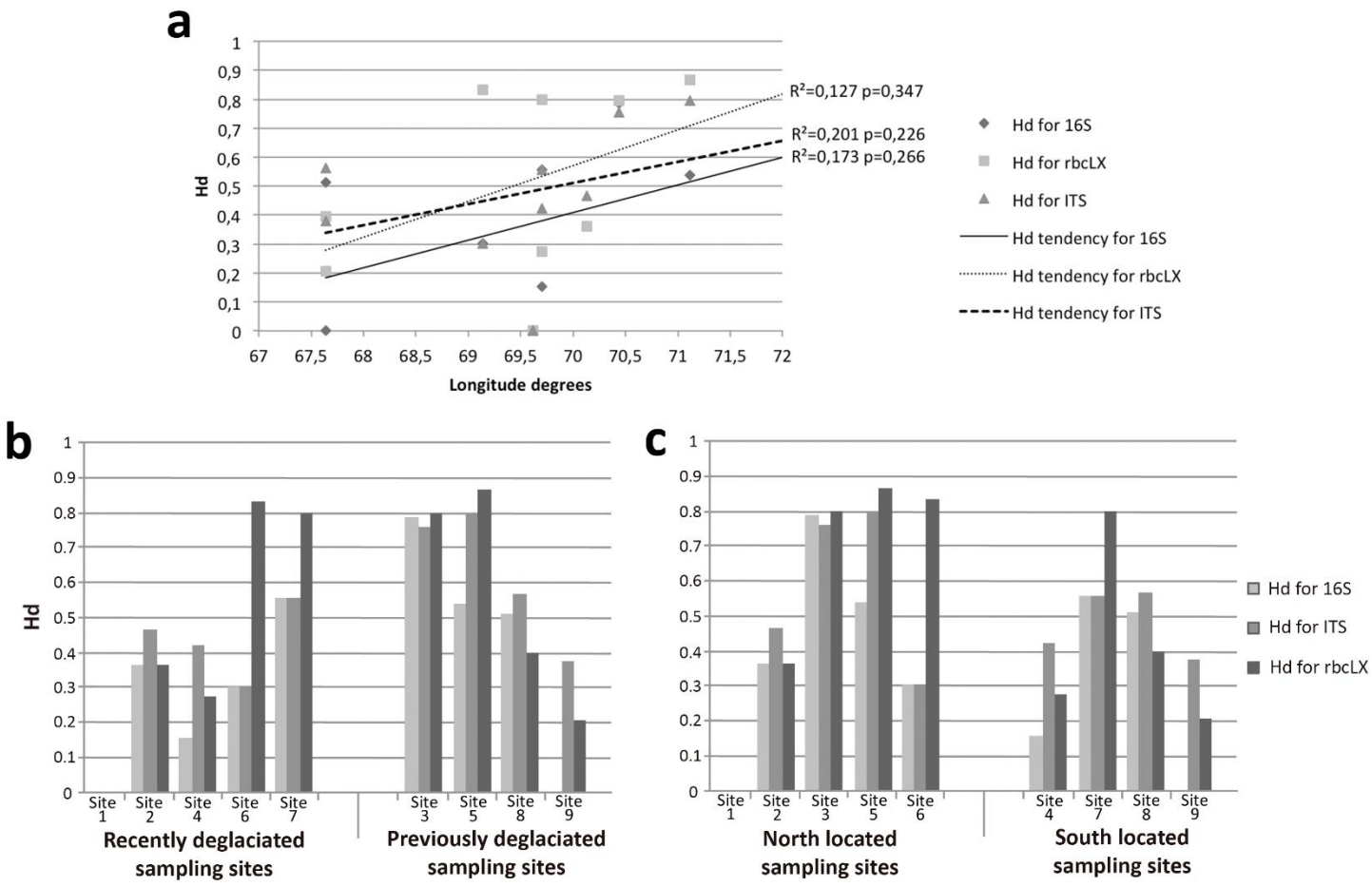
Table 3.3 Number of OTUs and the number of sequences in the most common OTU obtained under different dissimilarity cut-off levels.

OTUs	**Genomic region**		
% cut-off value	*16S*	ITS	*rbc*LX
No. of OTUs			
Unique	12	19	57 (32)
1%	4	19	38 (13)
2%	2	14	28 (7)
3%	2	12	23 (6)
5%	1	8	15 (4)
No. of seqs. In the most common OTU			
Unique	36	30	22 (28)
1%	49	30	25 (39)
2%	59	49	28 (47)
3%	59	50	30 (50)
5%	113	55	30 (50)

The haplotype distribution through the sampling area could reflect different environmental gradients. Fig. 3.5 shows the response of *Hd* values with respect to three environmental factors. Fig. 3.5a shows a clear trend of *Hd* increasing with longitude rise of the sampling areas for the three genomic regions, although low r^{2}`s and no significant p-values were found in all the cases. Evolution of *Hd* values for the three genomic regions in relation to time since ice retreat (Fig. 3.5b) and slope in the Cordillera Darwin (Fig. 3.5c) are also represented. Fig. 3.5b shows there is a trend of higher *Hd* values in areas of longer times since deglaciation, while in Fig. 3.5c higher *Hd* values correspond to Northern sites. A Mantel test showed relatively low correlation between geographic and genetic distances (0.125, p-value<0.01). Position concerning Cordillera Darwin was revealed as the most determining within the factors analyzed, with percentages of 92.5 and of 63.05 of variance explained (p-value<0.01) through AMOVA analyses for *16S* and for *rbc*LX gene regions, respectively (Table 3.4, North *vs*. South location). Other factors considered, such as the location of the sampling site in retreating glacer areas and the time since ice retreated, were less explicative trhough independent factor analyses (Table 3.4).

Table 3.4 Results from AMOVA testing for *16S* and *rbc*LX genomic regions.

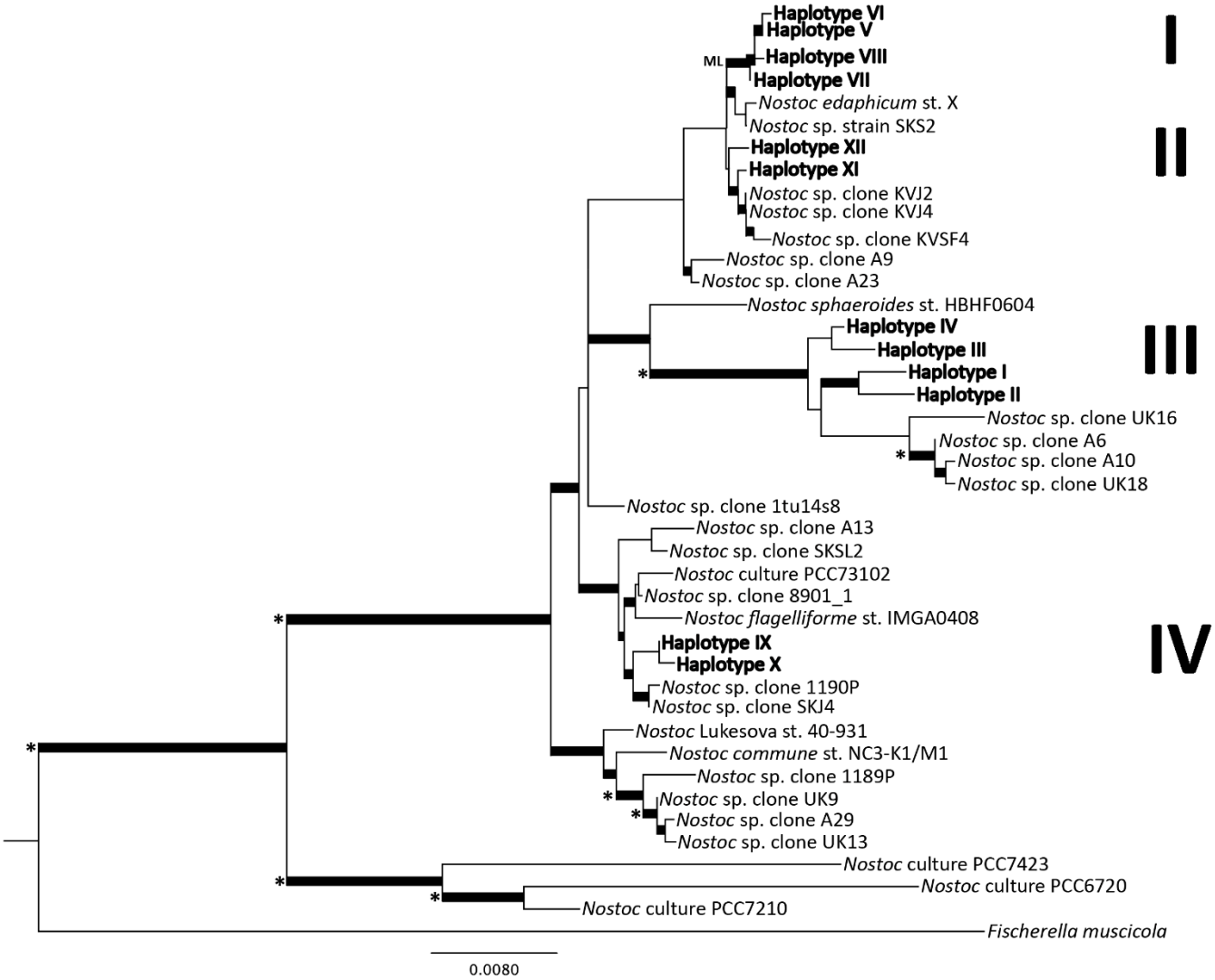
Genomic region	Analysed parameter	Percentage of variation explained		
		Among populations	Among populations within groups	Within populations
16S	Glacier settlement	54.54% (*p*=0.000)	33.02% (*p*=0.118)	19.02% (*p*=0.000)
	Time since ice retreat	60.90% (*p*=0.000)	28.32% (*p*=0.072)	21.02% (*p*=0.000)
	North vs. South location	92.50% (*p*=0.002)	10.69% (*p*=0.619)	25.24% (*p*=0.000)
*rbc*LX	Glacier settlement	49.90% (*p*= 0.000)	14.56% (*p*=0.218)	35.54% (*p*=0.000)
	Time since ice retreat	54.30% (*p*=0.000)	7.81% (*p*=0.208)	37.89% (*p*=0.000)
	North vs. South location	63.05% (*p*=0.005)	2.81% (*p*=0.429)	39.77% (*p*=0.000)



◀ **Fig. 3.5.** *Hd* values for sampling sites concerning the different environmental features taken into account in present study. **(a)** Variation in *Hd* values in response to Longitude coordinate of sampling sites. *Hd* values are represented for the three genomic regions: diamonds for *16S* values and solid line for *16S* tendency, squares for *rbc*LX values and dotted line for *rbc*LX tendency and triangles for ITS values and discontinuous line for ITS tendency. **(b)** *Hd* values for sampling sites, split into two groups showing sites in recently deglaciated areas (left) and previously deglaciated areas (right). *Hd* values are represented for the three genomic regions: light grey for *16S*, grey for ITS and dark grey for *rbc*LX. **(c)** *Hd* values for sampling sites, split into two groups showing northern sites (left) and southern sites (right). *Hd* values are represented for the three genomic regions: light grey for *16S*, grey for ITS and dark grey for *rbc*LX.

The *16S* phylogenetic tree showed haplotypes clustered into four different groups (Fig. 3.6). All these groups were relatively close to sequences of different known species retrieved from the GenBank database in BLAST searches. Thus, haplotypes V, VI, VII, VIII (group I) and haplotypes XI and XII (group II) were closely related to *N. edaphicum* (strain X); haplotypes I, II, III and IV (group III), together with other sequences obtained from Gen Bank, resulted in a group sister to *N. sphaeroides* (strain HBHF0604) and finally, haplotypes IX and X (group IV) were clustered in a supported clade that includes *N. flagelliforme* (strain IMGA0408). In majority of cases, the most closely related strains corresponded to sequences from uncultured Nostoc found in symbiosis with hepatics or lichen-forming cyanobacteria (bi- and tripartite lichens). related strains corresponded to sequences from uncultured *Nostoc* found in symbiosis with hepatics or lichen-forming cyanobacteria (bi- and tripartite lichens).

Fig. 3.6. Phylogenetic 50% majority rule tree for *16S* gene dataset. Lines in bold show branches that are supported in Bayesian analysis (PP>0.95); those showing ML are supported only in maximum likelihood analysis (BP>0.70); those marked with ∗ are supported in both analyses. Line under the tree represents substitutions per site scale. Roman numerals refer to posterior comments. ▶

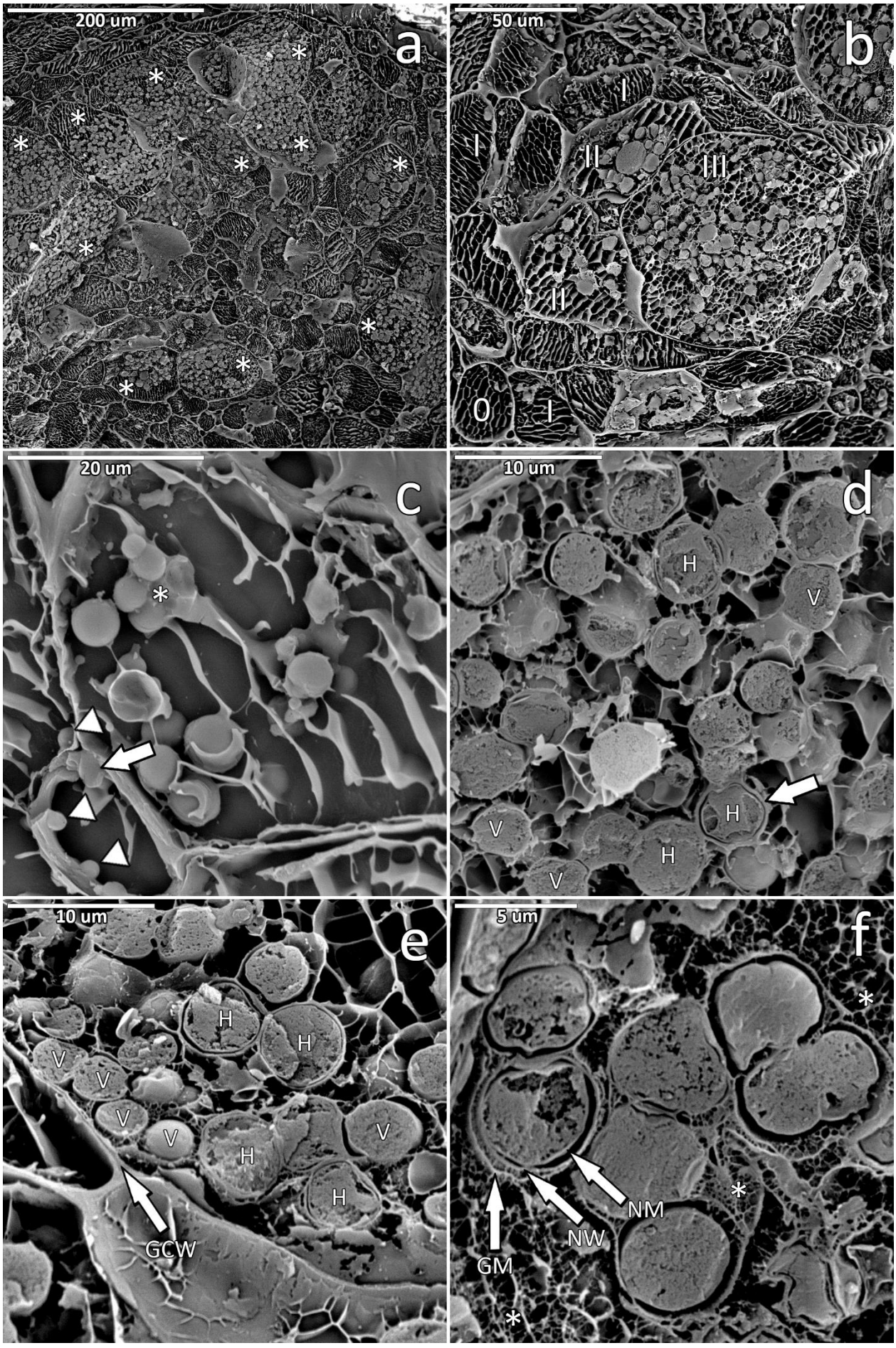


Phylogenetic analyses for *rbc*LX gene haplotypes (Fig. 3.7) showed that all the haplotypes were also clustered in four major groups, with 14, 10, 6 and 2 haplotypes, respectively. The four groups are closely related to *Nostoc* sequences obtained from other symbiotic associations (mainly of the cyanolichen genus *Peltigera*, especially for the second and third groups). These groups could not be easily assimilated within known *Nostoc* taxa. The first and second groups seem to be close to *Nostoc* known from lichen symbioses; the third group is also close to a symbiotic *Nostoc* from a lichen, but in this case to the photobiont of *Massalongia carnosa*, which appears far from the others in the analysis. Finally, the fourth group is likely close to these *Nostoc* from different lichens, but also to *N. flagelliforme*.


Haplotype 11
Haplotype 13
Haplotype 15
Haplotype 16
Haplotype 17
Haplotype 18
Haplotype 19
Haplotype 20
Haplotype 21
Haplotype 22
Haplotype 23
Haplotype 24
Haplotype 25
Haplotype 26
Nostoc sp. 'Peltigera sp. cyanobiont'
Nostoc sp. 'Mollenhauer 1:1-150b'
Nostoc sp. strain SAG 36.92
Nostoc muscorum strain SAG 57.79
Nostoc sp. 'Mollenhauer 1:1-088'
Nostoc sp. 'Peltigera lepidophora cyanobiont'
Nostoc sp. strain 1tu14S8
Nostoc sp. 'Mollenhauer 1:1-066'
Nostoc commune
Nostoc sp. 'Mollenhauer 1:1-064'
Nostoc sp. 'Mollenhauer 1:1-065'
Nostoc punctiforme strain SAG 71.79
Nostoc sp. 'Collema nigescens (245) cyanobiont'
Nostoc sp. 'Collema fragile (214) cyanobiont'
Nostoc sp. 'Leptogium pseudofurfuraceum (274) cyanobiont'
Nostoc sp. strain NIVA-CYA 194
Haplotype 2
Haplotype 6
Haplotype 5
Haplotype 9
Haplotype 10
Haplotype 4
Haplotype 1
Haplotype 7
Haplotype 8
Haplotype 3
Haplotype 27
Haplotype 30
Haplotype 31
Haplotype 32
Haplotype 28
Haplotype 29
Nostoc sp. 'Massalongia carnosa cyanobiont' clone H20961R
Nostoc sp. 'Peltigera leucophlebia cyanobiont'
Nostoc sp. 'Peltigera praetexata cyanobiont'
Nostoc sp. strain SAG 39.87
Nostoc sp. 'Peltigera membranacea 5 cyanobiont'
Nostoc sp. 'Peltigera horizontalis cyanobiont'
Nostoc sp. 'Peltigera membranacea 3 cyanobiont'
Nostoc sp. 'Peltigera membranacea 2 cyanobiont'
Nostoc sp. strain SAG 41.87
Nostoc sp. 'Mollenhauer 1:1-125'
Nostoc sp. 'Peltigera didactyla 3 cyanobiont'
Nostoc punctiforme st. SAG 68.79
Nostoc flagelliforme
Haplotype 12
Nostoc sp. 'Leptogium azureum (270) cyanobiont'
Haplotype 14
Nostoc sp. 'Leptogium pulvinatum (281) cyanobiont'
Nostoc sp. 'Leptogium pulvinatum (282) cyanobiont'
Nostoc sp. 'Collema auriforme (150) cyanobiont'
Nostoc sp. 'Leptogium corniculatum (131) cyanobiont'
Nostoc sp. 'Leptogium gelatinosum (280) cyanobiont'
Nostoc sp. 'Leptogium corniculatum (130) cyanobiont'
Nostoc sp. 'Collema tenax (248) cyanobiont'
Nostoc sp. 'Collema subnigrescens (285) cyanobiont'
Nostoc sp. 'Collema furfuraceum (298) cyanobiont'
Nostoc sp. 'Collema furfuraceum (1009) cyanobiont'
Nostoc sp. 'Leptogium brebissonii (161) cyanobiont'
Nostoc sp. 'Collema nigrescens (221) cyanobiont'
Nostoc sp. 'Lobaria pulmonaria cyanobiont'
Nostoc sp. 'Nephroma parile cyanobiont'
Nostoc sp. 'Pseudocyphellaria crocata cyanobiont' clone W090127R
Nostoc sp. 'Leptogium lichenoides (328) cyanobiont'
Fischerella muscicola culture PCC 7414
ML
*
1.0

◀**Fig. 3.7.** Phylogenetic 50% majority rule tree for *rbc*LX gene with collapsed haplotypes/OTUs after Gblocks trimming. Lines in bold show branches that are supported in Bayesian analysis (PP>0.95); those showing ML are supported only in maximum likelihood analysis (BP>0.70); those marked with ∗ are supported in both analyses. Line under the tree represents substitutions per site scale. "Haplotype 1" also comprises haplotypes/OTUs 33, 34, 35, 49 and 50; "Haplotype 2" comprises also haplotypes/OTUs 41, 52 and 53; "Haplotype 11" comprises also haplotypes/OTUs 36, 7, 38, 46, 47, 48, 54, 55 and 56; "Haplotype 12" also comprises haplotype/OTU 39; "Haplotype 26" also comprises haplotype/OTU 57; "Haplotype 27" also comprises haplotypes/OTUs 40 and 42; "Haplotype 31" also comprises haplotypes/OTUs 43, 44, 45 and 51. Roman numerals are only for easier interpretation of posterior comments.

The biotrophic interface between symbiotic *Nostoc* and *G. magellanica* cells was analyzed by LTSEM (Fig. 3.8). Infected *Gunnera* cells were numerous with larger size and more rounded shape (Fig. 3.8a) than uninfected ones (0 in Fig. 3.8b). This was especially noteworthy in later stages of infection (III in Fig. 3.8b). Earliest stages (I in Fig. 3.8b) are characterized by the intracellular location of a few *Nostoc* cells in some host cells. Penetration of small *Nostoc* cells through host cell wall was detected at these stages (arrow in Fig. 3.8c), which may represent first steps of infection. Microsymbiont cells were generally observed positioned close to plant cell wall (arrowheads in Fig. 3.8c). *Nostoc* cells appeared surrounded by host plasma membrane and embedded in a matrix of extracellular polymeric substances (EPS) (asterisk in Fig. 3.8c). In later stages of infection (II and III in Fig. 3.8b), host cells appeared colonized by filaments of *Nostoc* vegetative cells with numerous heterocysts (Fig. 3.8d and 3.8e). These latter cells were distinguishable by their larger size and a slightly thickened cell wall. Filaments containing heterocysts appeared embedded in an EPS matrix (asterisks in Fig. 3.8f).


200 um
a
50 um
b
I
II
III
0
20 um
c
10 um
d
H
V
10 um
e
GCW
5 um
f
NM
NW
GM

◀ **Fig. 3.8.** LTSEM images of *Gunnera magellanica* tissue infected with *Nostoc*. **(a)** Infected root tissue of *G. magellanica*; *Gunnera* cells containing *Nostoc* cells (asterisks) are larger than uninfected ones, also shown; **(b)** Area of root showing plant cells in different stages of infection by *Nostoc* cells. "0": uninfected plant cell; "I": plant cells at earliest stages of infection, with few *Nostoc* cells inside them; "II": intermediate stage of infection, with larger size and higher number of symbionts; "III": plant cell at mature infection stage, showing largest more numerous symbionts within it and with well-developed EPS matrix. **(c)** Infected *Gunnera* cell at an early stage of infection. Asterisk marks a filament of vegetative *Nostoc* cells embedded in EPS matrix. Arrowheads point to *Nostoc* symbiotic cells close to plant cell wall while arrow indicates *Nostoc* cell penetrating through host cell wall (first steps of infective processes). **(d)** Plant cell at a later infection stage (III), containing numerous filaments of *Nostoc* vegetative cells (V) with numerous heterocysts (H) distinguishable by larger size and thickened cell wall. Arrow points to the heterocysts cell wall showing thickness. **(e)** Infected host cell showing numerous *Nostoc* filaments in the proximity of its cell wall. Filaments of small vegetative cells are observed closely associated to the host cell wall (arrowhead). Filaments containing heterocysts (H) were located inwardly. GCW: *Gunnera* cell wall. **(f)** Detail of an infected host cell at an intermediate stage of infection (II), showing *Nostoc* cells bounded by host cell plasma membrane and associated with EPS fibers (asterisks). Arrows point to the three layers sourrounding *Nostoc* cell inside the host cell: *Gunnera* plasma membrane (GM), *Nostoc* cell wall (NW) and *Nostoc* membrane (NM).

3.4 Discussion

Results showed that every *G. magellanica* endosymbiont analyzed belongs to the genus *Nostoc*, but different species are involved in the *Gunnera-Nostoc* symbioses studied. This is consistent with previous studies that identified *Nostoc* as symbiont of *Gunnera* (Bergman et al. 1992; Bergman 1996; Rai et al. 2000; Rasmussen and Svenning 2001; Svenning et al. 2005), including the South Chilean host species used in the present study, *G. magellanica*, and the nearby *G. tinctoria* (Bonnet and Silvester 1981; Guevara et al. 2002). However, previous studies included only a few specimens of *G. magellanica*. The extensive sampling of different colonies in the same *Gunnera* specimen and in different specimens from diverse sites provided a better perspective on symbiont diversity at different levels and also some clues about the factors influencing these

diversity values. The phylogenetic position of the different *Nostoc* symbionts found in *G. magellanica* species has been analyzed for the first time.

No genetic variability was detected in *Nostoc* sampled from the same host specimen of *G. magellanica* (sequences from 15 plants). Following these results, it is possible to state that each plant was likely infected by a single strain. These results agree with those obtained in different species of *Gunnera* using fingerprinting technique (Guevara et al. 2002). The lack of intra-specimen variability has also been reported for other *Nostoc* symbiotic associations, especially those formed with bryophytes or lichen-forming fungi (Paulsrud and Lindblad 1998; Costa et al. 2001). Bonnet and Silvester (1981) found similar conclusions by means of *Nostoc* inoculation assays in *Gunnera* plants, suggesting further that not every strain is able to infect every plant. Nevertheless, Nilsson *et al.* (Nilsson et al. 2000) isolated more than one strain from one single infection in *Gunnera*, identifying them by using STRR-PCR fingerprinting on cultures. Thus, present results and those obtained in cited studies point at a process of selection of symbiont strains by the plant that could occur at a very early stage of the infection process. This early selection might inhibit a second infection by other strains of cyanobacteria (Meeks and Elhai 2002).

By contrast, genetic diversity analysis showed high intra- and intersite variability of *G. magellanica Nostoc* symbionts in Tierra del Fuego, in spite of the short distance between the easternmost and the westernmost sites (c. 350 km). Our results showed a genetic diversity gradient from the most internal sampling areas (central part of Isla Grande de Tierra del Fuego) to the most external ones (areas near oceanic coast) (Figs. 3.1, 3.2, 3.3). Sites with lower haplotype diversity values (coincident results for the three studied genomic regions) were those where glacier retreat occurred most recently (since the later Late Glacial period, 10 ka) (Holmlund and Fuenzalida 1995; Rabassa et al. 2011). On the other hand, higher haplotype diversity values were found in sites close to areas where ice disappeared in earlier times (Last Glacial Maximum and first Late Glacial periods, 20-15 ka) (Holmlund and Fuenzalida 1995; Rabassa et al. 2011). This tendency is also shown in Fig. 3.5b, despite uneven *Hd* values for different sites. Similar results were found by Nemergut et al. (2007) in a glacier foreland of the Peruvian Andes. Easternmost sites *8* and *9* (located in very close forest and tundra areas) did not show high haplotype diversity values as expected from the length of time since ice retreat

(Rabassa et al. 2011) and the presence of stable vegetation in the area (Markgraf and Huber 2010). The lower diversity observed at these sites might be more influenced by the low precipitation values for both sites, and the tundra environment conditions in *Site 9* (Koppes et al. 2009). High precipitation values may benefit the development of both *Nostoc* and *G. magellanica* because of their physiological requirements (Wilkinson and Wanntorp 2007), and this could favor the *Nostoc* symbiotic diversity as well as hormogonia (infective filaments) development (Herdman 1988; Gantar et al. 1993). This development could drive to a more intense infection processes of all competent *Nostoc* strains of the area. In fact, higher degrees of *Nostoc* diversity was indeed found in sampling sites *3, 4, 5* and *7*, areas where precipitation is more abundant. This precipitation regime is due to proximity of the Pacific Ocean (Holmlund and Fuenzalida 1995; Koppes et al. 2009) and southern exposure on a slope of Cordillera Darwin (Molina 1983; Koppes et al. 2009), as well as more stable surrounding vegetation (Markgraf and Huber 2010). These results agree with AMOVA testing for independent factors, which showed that location with respect to the Cordillera Darwin was the factor that most explained the genetic variance (Table 3.4). Moreover, low correlation between geographic and genetic distances, showed by means of a Mantel test, suggests that diversity of *Nostoc* strains associated to *G. magellanica* at each site is influenced not only by the geographic position but also by a combination of several factors.

Regarding the presence or absence of different symbiotic *Nostoc* strains in each sampling site, it is noteworthy that while some of haplotypes were present only in sampling sites furthest from the ocean (where ice-retreatment happened later), others were only shared among sites close to the ocean. This could mean that specific *Nostoc* strains might be predominant in the early stages after glacial ice retreat, as was found in analyses of *NifH* gene sequences in a retreating alpine glacier (Duc et al. 2009). Further investigations of *Nostoc* haplotypes from soil and in symbiosis with *G. magellanica*, including Fuegian samples as close as possible to glacier ice might help to test this fact.

The finding of two OTUs by the analysis of the *16S* region of ribosomal RNA gene using a dissimilarity cut-off value of 0.03 (Martínez-Murcia and Collins 1990; Collins et al. 1991; Amann et al. 1992; Fox et al. 1992; Martínez-Murcia et al. 1992) may indicate the presence of two distinct species. Moreover, following Hart and Sunday (2007), the existence of two isolated networks of haplotypes with a 95% parsimony probability

criterion, might point to the presence of two species as well. However, four different taxa would be distinguished following the assumptions of Stackebrand and Ebers (2006): these latter authors, based on data correlation between sequence similarity and DNA-DNA reassociation experiments, considered that for prokaryotes a cut-off value of 99% is required to discriminate between two species. In fact, phylogenetic analysis (for *16S* and also for *rbc*LX) showed that haplotypes analyzed were clustered in four groups (Fig. 3.6, 3.7). At a higher taxonomic level (using a cut-off value of 0.05, following Martínez-Murcia and Collins 1990; Collins et al. 1991; Fox et al. 1992; Martínez-Murcia et al. 1992) all the *16S* gene sequences were included in one group, indicating the presence of only the genus *Nostoc*.

The clusterings obtained by *rbc*LX sequences (without any trimming of ambiguously aligned regions) and ITS ones were equivalent to that obtained for *16S* one only when lowest cut-off values were used. The two groups resulted from *16S* sequences using a 3% dissimilarity cut-off could also be obtained for both ITS and *rbc*LX genomic regions using cut-off values of approximately 20% dissimilarity. Indeed, these groups for *16S,* ITS and *rbc*LX were composed of sequences obtained from the same specimens. Rudi et al. (1998) found that, depending on the *Nostoc* lineage of interest, *rbc*LX region sequences divergence displays a 2 to 35-fold-higher difference compared to *16S*. With this taken into account, our results may point to the possibility of using ITS and *rbc*LX genomic regions not only for populational studies (García-Martínez et al. 1999; Han et al. 2009), but also for taxonomic purposes. Further analyses using a broad range of cyanobacterial groups are necessary to determine the optimum cut-off values for delimiting taxonomic groups by using sequence similarity in ITS and *rbc*LX regions, extending the criteria beyond *16S* results.

Nostoc haplotypes obtained in the present study have been shown to be closely related to *N*. *edaphicum*, *N. sphaeroides,* and *N. flagelliforme* by analysis of *16S* sequences. A correspondence to *N. flagelliforme* was also found in *rbc*LX analysis; this *Nostoc* species has been found before in symbiosis with other *Gunnera* species (Svenning et al. 2005). It is remarkable that many of our haplotypes showed close relationship to sequences from symbiotic *Nostoc* of different groups of organisms found in the northernmost areas of Europe. For instance, the sequences clustering with *N*. *edaphicum* were related to the *Nostoc* strains SKS2, KVJ2 or KVJ4, found in hepatics

(retrieved from the GenBank database, not published), while those clustering with *Nostoc flagelliforme* were related to cyanolichen symbionts from China (Svenning et al. 2005).

The intracellular position of symbiotic *Nostoc* cells within *Gunnera magellanica* has been confirmed using LTSEM. This observation was in agreement with what other authors have found using other microscopy techniques, such as TEM (Silvester and McNamara 1976; Towata 1985). As far as we are aware, the *Nostoc*-*Gunnera* symbiosis has not been previously examined with LTSEM. This technique allowed us to visualize the EPS matrix in which filaments of *Nostoc* cells are immersed through the infection process. Penetration of *Nostoc* cells through host cell walls and location outside the host plasma membrane could also be observed and different stages of infection characterized. The infection started with the penetration of small vegetative cells through the host cell wall (I); in later stages (II and III) filaments of *Nostoc* containing vegetative and heterocysts cells progressively occupied the entire host cell, as a host cell size increasing. All morphological and ultrastructural features were in agreement with those described in previous TEM studies (Silvester and McNamara 1976; Han et al. 2009). Further observations with this technique at different stages of host colonization by *Nostoc* cells could increase our understanding of infection and symbiotic *Nostoc* differentiation processes.

From the results obtained in this study we conclude that an individual *Gunnera magellanica* specimen takes up and hosts only one *Nostoc* strain. Although the *Nostoc* genetic diversity values can vary when using different markers (*16S*, ITS or *rbc*LX regions), we found a maximum of four species of *Nostoc* occurring in symbiosis with *Gunnera magellanica* in the populations analyzed. The span of time for which soils have been exposed to environmental conditions and the precipitation regime were recognized as the two prime factors influencing the values of diversity of symbiotic *Nostoc* in *Gunnera magellanica* specimens from distinct sites in Tierra del Fuego. Through symbiosis, *Nostoc* may be extending its ecological niche sheltered from external predators. In this way, pioneer strains might persist in certain localities while new colonizations increase the diversity of *Nostoc* recognizable species by means of a dynamic process. Further studies comparing the diversity of *Nostoc* strains present in

soils to those found in *Gunnera* plants are necessary to assess the degree of selectivity in this cyanobacteria-angiosperm symbiosis.

DISCUSIÓN GENERAL

"El tiempo no es oro: el oro no vale nada.

El tiempo es vida"

José Luis Sampedro

E. DISCUSIÓN GENERAL

Los suelos descubiertos por el retroceso de glaciares en Tierra del Fuego presentan una extensiva colonización microbiana por parte de bacterias, hongos, algas arqueas y virus. Estos microorganismos, componentes mayoritarios en las etapas iniciales de la sucesión, y parte importante de las comunidades más complejas de etapas posteriores, muestran una gran diversidad funcional que cambia a lo largo del proceso sucesional en concordancia con los cambios en su diversidad taxonómica. El uso conjunto de diferentes técnicas de estudio, tal y como ha sido sugerido con anterioridad por otros autores (Schuster 2008; Zhou 2009; Hirsch et al. 2010; Loman et al. 2012; Zhou et al. 2015), ofrece una amplia perspectiva de esta diversidad taxonómica y funcional de las comunidades microbianas, lo que ha permitido caracterizar con gran detalle los procesos de sucesión en estos terrenos descubiertos tras el retroceso de glaciares de la región chilena. Los resultados de esta tesis doctoral sugieren que la estructura de las comunidades microbianas no depende solo del tiempo que llevan deglaciados estos terrenos, mostrando a su vez patrones similares a los descritos en zonas semejantes de otras latitudes, tanto del Hemisferio Sur (Nemergut et al. 2007; Bajerski y Wagner 2013; Ciccazzo et al. 2015), como aquellas, mucho más estudiadas, del Hemisferio Norte (Kandeler et al. 2006; Bardgett et al. 2007; Hawes 2008; Schutte et al. 2009, 2010; Brankatschk et al. 2011; Wu et al. 2012; Zumsteg et al. 2012; Frey et al. 2013; Schulz et al. 2013; Brown y Jumpponen 2014; Ciccazzo et al. 2015). Sin embargo, la dinámica de sucesión microbiana (*i.e.* tasas anuales de recambio de taxones y cambios en la diversidad de genes funcionales entre etapas sucesionales) en estos glaciares de Tierra del Fuego es más rápida que las descritas para la gran mayoría de áreas deglaciadas.

E.1 Diversidad taxonómica y funcional de las comunidades microbianas a lo largo de las cronosecuencias

Los suelos de las etapas iniciales de la sucesión de los glaciares de Tierra del Fuego estudiados, Pía y Parry, se caracterizan por presentar una baja disponibilidad de nutrientes (*v.* Arróniz-Crespo et al. 2014), así como un escaso grado de desarrollo

(Thébault et al. 2014). Los microorganismos presentes en estos suelos pueden haber sido transportados por el aire, desde áreas más o menos limítrofes o desde capas superiores del propio glaciar, así como a través del agua de deshielo, pudiendo también haber sido depositados por las precipitaciones en forma de lluvia o nieve (Foght et al. 2004; Kastovská et al. 2005; Hodson et al. 2008; Takeuchi 2011). Pese al potencial de colonización de los suelos de estas áreas por parte de un gran número de microorganismos diferentes, nuestros resultados apuntan a que sólo aquellos cuyo metabolismo les permite establecerse en condiciones oligotróficas pueden desarrollarse en estas primeras etapas, tal y como ha sido descrito en otros terrenos de glaciares en retroceso (Sigler y Zeyer 2004; Frey et al. 2013). Junto a estos microorganismos pioneros será posible encontrar formas de resistencia de otras especies, así como aquellos microorganismos cuya presencia sea sólo circunstancial (Miteva et al. 2004; Sigler y Zeyer 2004; Kastovská et al. 2005). Sin embargo, con las herramientas utilizadas en nuestro trabajo es imposible discriminar entre formas durmientes y activas de las organismos encontrados.

De entre los grupos taxonómicos pioneros encontrados en estas etapas iniciales de sucesión destacan, por su abundancia y función, las *Actinobacteria*, las cuales aparecieron en los análisis como estadísticamente ligadas a estos estadios. De acuerdo a los resultados extraídos del análisis con GeoChip, este grupo bacteriano presenta la capacidad de degradar diferentes compuestos orgánicos (tanto lábiles como recalcitrantes). Las *Actinobacteria* presentes en estos suelos parecen estar participando en procesos de degradación de los escasos compuestos orgánicos existentes en los suelos más próximos al frente glaciar, un hecho anteriormente propuesto en varios estudios (Heuer et al. 1997; Vetrovsky et al. 2014). Parte de la materia orgánica que estas bacterias estarían degradando podría haber sido generada en épocas anteriores al desarrollo de la capa de hielo, quedando atrapada bajo ella (como se propuso en Bardgett et al. 2007) tras avances glaciares como los que se han producido en esta área durante la Pequeña Edad de Hielo (Davies y Glasser 2012; Melkonian et al. 2013).

En los suelos más cercanos al frente glaciar destacó también la presencia de la clase *Cyanobacteria*, grupo escasamente representado en otras etapas sucesionales posteriores. Las cianobacterias tienen la capacidad de fijar carbono y nitrógeno atmosférico, lo que les confiere ventajas competitivas en áreas que, como éstas,

presenten una disponibilidad de nutrientes muy baja (Kastovská et al. 2005; Schimdt et al. 2008; Frey et al. 2013). Nuestros resultados apuntan a que gracias a su metabolismo fotosintético y diazotrófico, las cianobacterias, junto con varios grupos de algas detectados también en estos suelos (el orden *Prasiolales* parece ser especialmente abundante) podrían ser elementos clave en estas etapas iniciales, de manera semejante a lo propuesto en otras áreas glaciares (Kastovská et al. 2005; Frey et al. 2013). Sin embargo, los genes *nifH* detectados por GeoChip, los cuales codifican para enzimas implicadas en la fijación del nitrógeno atmosférico (Duc et al. 2011), no correspondieron mayoritariamente a taxones de *Cyanobacteria*, tal y como cabría esperar por la abundancia de este grupo bacteriano. En cambio, la capacidad de fijar nitrógeno atmosférico en estas primeras etapas estuvo asociada a otros grupos microbianos no fotosintéticos, principalmente a las *Alphaproteobacteria*. Estos grupos de organismos diazótrofos parecen tener una gran importancia para los procesos de sucesión posteriores gracias a su potencial introducción de nitrógeno en el medio (Schimdt et al. 2008; Zumsteg et al. 2012). Otras actividades metabólicas potenciales detectadas para *Cyanobacteria* dentro del ciclo del nitrógeno, como la amonificación, podrían ser también de gran importancia en estas etapas iniciales. Además, estas actividades potenciales fueron también encontradas en grupos como *Chloroflexi* y *Deinococcus-Thermus*. Así, altas tasas de amonificación, junto con procesos de fijación de nitrógeno, generarían el sustrato necesario para que procesos de nitrificación puedan tener lugar en estos suelos (Fierer et al. 2010; Zumsteg et al. 2012; Brankatschk et al. 2013; Frey et al. 2013; Bradley et al. 2014). Los resultados combinados de diversidad taxonómica y funcional mostraron que la nitrificación podría ser llevada a cabo por bacterias principalmente de distintos grupos de *Proteobacteria*, incluyendo la clase *Epsilonproteobacteria*, la cual, si bien no es muy abundante en estas zonas, sí muestra una alta señal en los análisis mediante GeoChip para los genes que codifican enzimas implicadas en nitrificación (*e.g. amoA*, *hao*).

La composición taxonómica, así como las identidades de los genes funcionales detectados con mayor frecuencia en estas etapas iniciales de sucesión, presentaron grandes diferencias con las etapas sucesionales posteriores. Los altos valores encontrados en las tasas de recambio de taxones (especialmente de bacterias y algas) entre las etapas iniciales y las inmediatamente posteriores en los dos glaciares

analizados (4 y 18 años de exposición del suelo en las vertientes sur y norte, respectivamente) indican que gran parte de los microorganismos detectados en etapas iniciales son reemplazados por nuevos colonizadores, capaces de desarrollarse más eficientemente en los nuevos microambientes que se generan en el suelo, de manera semejante a lo encontrado en otros procesos sucesionales en terrenos deglaciados (Skidmore et al. 2005; Bernasconi et al. 2011; Bajerski y Wagner 2013; Schulz et al. 2013; Bradley et al. 2014). Un claro ejemplo de los cambios drásticos entre las etapas iniciales de la sucesión y el resto es la ausencia de miembros de las clases *Cyanobacteria* y *Chytridiomycetes* más allá de las primeras etapas.

Con la colonización por parte de briófitos y líquenes (de los Ríos et al. 2011, Arróniz-Crespo et al. 2014) comienzan una serie de estados de sucesión intermedios. En estas etapas dominadas por criptógamas se encontró una alta señal de genes *nif* para distintos grupos de *Proteobacteria*, junto con *Chlorobi* y *Chrysiogenetes*. Tras el establecimiento de las citadas comunidades de criptógamas, destaca el asentamiento que llevan a cabo plantas de *Gunnera magellanica*, considerada una especie vegetal pionera por la capacidad que presenta de asentarse en suelos con escasa concentración de nutrientes, gracias a la formación de simbiosis con células de *Nostoc* (Guevara et al. 2002). Estas plantas contribuyen a la acumulación en el medio de materia orgánica, incluyendo compuestos nitrogenados, originados principalmente a partir del nitrógeno fijado por los simbiontes de *Gunnera magellanica*. De hecho, se ha detectado un incremento notable de las concentraciones, tanto de amonio, como de otros compuestos inorgánicos de nitrógeno tras su establecimiento (datos en Arróniz-Crespo et al. 2014), tal y como ocurre en terrenos colonizados por *Alnus* en simbiosis con *Frankia* (Crocker y Major 1955; Chapin et al. 1994; Schwencke y Carú 2001; Walker y del Moral 2003; Chaia et al. 2010; Anderson et al. 2013). A su vez, el desarrollo de las comunidades de *G. magellanica* disminuye la compactación de los horizontes superficiales del suelo (Thébault et al. 2014), favoreciendo posteriores colonizaciones de vegetación vascular, como especies de la familia *Ericaceae* (*Empetrum rubrum* y *Gaultheria mucronata*).

Las diferentes potencialidades para rutas metabólicas de los ciclos del nitrógeno y carbono, inferidas mediante GeoChip, en comunidades microbianas de etapas sucesionales intermedias consecutivas, parecen reflejar actividades secuenciales dentro

de los ciclos de nutrientes del suelo. El nitrato acumulado en fases iniciales de sucesión por procesos de fijación, nitrificación y amonificación podría servir como sustrato de actividades metabólicas microbianas como la reducción desasimilatoria y asimilatoria de nitrato, y la desnitrificación, cuyos potenciales son detectados en estas etapas de sucesión consecutivas. Las capacidades para la reducción desasimilatoria y asimilatoria del nitrato fueron asociadas a grupos bacterianos muy abundantes en estos suelos, tales como *Alpha-*, *Beta-* y *Deltaproteobacteria*, junto con otros menos abundantes como *Deinococcus-Thermus*, y arqueas pertenecientes a *Euryarchaeota*. Por su parte, las potencialidades para procesos de desnitrificación fueron atribuidas a *Actinobacteria*, muy abundantes en estos suelos, o a taxones del grupo termófilo menos abundante *Aquificae* (Chèneby et al. 2000; Rösch et al. 2002; Palmer y Horn 2012; Shapleigh 2013).

En el caso de la movilización de compuestos de carbono, el análisis mediante el GeoChip detectó potencial para actividades acetogénicas en las etapas sucesionales intermedias donde ocurre la principal colonización por parte de líquenes y briófitos (4 años de exposición), estando asociada esta actividad principalmente a *Euryarchaeota* y *Deltaproteobacteria*. La detección de un elevado potencial para actividades metanogénicas en la etapa inmediatamente posterior (7 años de exposición) parece indicar que los grupos con potencial acetogénico podrían estar generando el sustrato para las reacciones metabólicas de grupos microbianos con capacidades metanogénicas (Hunger et al. 2015; Lever 2016). En concreto, el potencial para la metanogénesis fue detectado principalmente en arqueas de los grupos *Korarchaeota* y *Crenarchaeota*, y bacterias de los grupos *Epsilonproteobacteria*, *Bacteroidetes* y *Gammaproteobacteria*, siendo las últimas especialmente abundantes en esta etapa, a tenor de los resultados obtenidos en el estudio de pirosecuenciación. La asociación detectada entre el potencial para actividades de oxidación de metano (encontrado principalmente en *Betaproteobacteria* y *Actinobacteria*) y la fase sucesional siguiente (10 años de exposición) apunta a que se puede haber generado metano previamente, el cual serviría de sustrato para estas actividades oxidadoras. Los grupos microbianos que participan en el ciclo del metano requieren de unos ambientes muy específicos para realizar las actividades secuenciales detectadas, los cuales varían desde condiciones aerobias a anaerobias estrictas (Aronson et al. 2013; Levy-Booth et al. 2014; Chiri et al. 2015; Lever 2016). La colonización por criptógamas y plantas pioneras puede favorecer la creación

de estos distintos microambientes, incluso en proximidad, debido a la gran heterogeneidad existente en el suelo, generada en parte por los efectos de estas actividades metabólicas en el medio, tal y como han sugerido otros autores (Atlas y Bartha 2002; Yin et al. 2002; Giri et al. 2005; Voroney 2014). La formación de ambientes anaerobios en estos terrenos puede estar también asociada a la presencia de encharcamientos periódicos transitorios.

En etapas sucesionales avanzadas (*e.g.* a partir de 19 años de exposición en el Glaciar Pía y de 26 en el Parry) destaca la abundancia de grupos microbianos que hacen posible la degradación de la materia orgánica acumulada. Entre ellos destaca la presencia de grupos fúngicos saprótrofos, como la clase *Agaricomycetes*. De igual manera, en estas etapas avanzadas de la sucesión se han detectado genes implicados en rutas metabólicas de degradación de compuestos orgánicos complejos, tales como la degradación de ácidos grasos mediante el ciclo del glioxilato o la de compuestos recalcitrantes como terpenos o compuestos aromáticos por parte de grupos de *Proteobacteria* y grupos adscritos a la clase menos abundante *Actinobacteria*. La presencia únicamente en fases avanzadas del Glaciar Parry de grupos específicos de hongos y algas, tales como la clase *Archaeorhizomycetes* y el orden *Desmidiales*, respectivamente, podría indicar la influencia de las diferentes condiciones climáticas imperantes en cada una de ellas, las distintas dinámicas de sucesión y el diferente grado de modificación del suelo por parte de los organismos establecidos en etapas previas.

E.2 Dinámicas de la sucesión primaria en Tierra del Fuego

Los resultados de esta tesis apuntan a que las bacterias juegan un papel fundamental en las primeras etapas de la colonización, tras la retirada de los glaciares de Tierra del Fuego, para ser posteriormente los hongos, en etapas más avanzadas de la sucesión, los que presenten las principales influencias sobre el proceso sucesional, de forma similar a lo descrito en otros estudios (Hodge et al. 2000; Bardgett y Walker 2004; Knelman et al. 2012; Thébault et al. 2014; Cao et al. 2015; Zilla et al. 2015). La mayor variedad y versatilidad de las actividades metabólicas bacterianas permiten que las bacterias sean más eficaces en la colonización de terrenos recién descubiertos, con una disponibilidad de nutrientes limitada (Zumsteg et al. 2012; Vetrovsky et al. 2014). La

presencia de hongos en los suelos, sin embargo, se ve condicionada por su metabolismo heterótrofo, el cual requiere una acumulación previa de nutrientes, asociada en muchos casos a la colonización y actividad anterior de organismos autótrofos, y al subsiguiente asentamiento de plantas (Brown y Jumpponen 2014; Thébault et al. 2014), procesos descritos a lo largo de las cronosecuencias analizadas de Tierra del Fuego. Este hecho explica, a su vez, las menores tasas de recambio encontradas para los hongos en las primeras etapas de la sucesión, ya que hasta que esta acumulación de nutrientes se produce, son muy pocos los grupos fúngicos que pueden participar en los procesos sucesionales (Brown y Jumpponen 2014). Por su parte, la gran ubicuidad que presentan las algas a lo largo de todos los terrenos estudiados, más allá de la asociación de los órdenes *Prasiolales* y *Desmidiales* con las etapas sucesionales iniciales (cercanas al frente del glaciar) y más avanzadas (zonas boscosas), respectivamente, parece apuntar a una menor influencia de las mismas en la sucesión que la de bacterias y hongos. Esta detección ubicua de los diferentes grupos de algas podría también deberse a la presencia de numerosas formas no activas de los mismos, tal y como ha sido descrito en otros estudios (Kastovská et al. 2005; Stibal et al. 2006; Lennon y Jones 2011)

El estudio simultáneo realizado en esta tesis de dos glaciares en retroceso en áreas situadas en dos vertientes diferentes de la Cordillera Darwin, junto con el análisis de la diversidad de simbiontes en *Gunnera magellanica* en áreas de las dos vertientes y en zonas con distinto desarrollo del suelo, nos ha permitido inferir el efecto de diferentes condiciones climáticas sobre las dinámicas sucesionales. Así, se ha visto que las cronosecuencias de ambas vertientes comparten patrones de sucesión para cada uno de los grupos taxonómicos analizados, con ubicuidad para algunos taxones y especificidad para una etapa sucesional concreta para otros. Sin embargo, la variación de la estructura de las comunidades respecto al tiempo de exposición difiere entre las cronosecuencias de las dos vertientes, debido a que presentaron distintas dinámicas temporales de sucesión. Así, el recambio de taxones se produce de manera mucho más rápida en la vertiente sur que en la norte, de manera similar a lo encontrado para las tasas de desarrollo del suelo y la sucesión vegetal (Sancho et al. 2011; Arróniz-Crespo et al. 2014). La situación respecto de la Cordillera Darwin fue también el factor más influyente en relación al patrón de filotipos de *Nostoc* endosimbiontes de las distintas poblaciones de *Gunnera magellanica* estudiadas. Los valores relativos a factores como

el grado de humedad, periodicidad y abundancia de precipitaciones, *etc.*, se han considerado a menudo como los factores más importantes que permiten o dificultan el mejor desarrollo de las comunidades microbianas edáficas (Herdman y Ripkka 1988; Wilkinson y Wanntorp 2007; Zeglin et al. 2013). Por esto, más allá del tiempo de exposición, las marcadas diferencias de precipitación que se dan en diferentes áreas de Tierra del Fuego, parecen estar influyendo también en los procesos de colonización de las áreas descubiertas por retroceso de glaciares. Otros factores, tales como un mayor tiempo de exposición a luz solar dependiendo de la orientación de los terrenos colonizados, pueden tener también influencia en las diferencias en las dinámicas de la colonización observadas (Sigler y Zeyer 2002; Wang et al.2010; Bajerski y Wagner 2013).

Independientemente de la velocidad de los procesos de sucesión, el recambio de especies en ambas cronosecuencias analizadas conduce a etapas sucesionales caracterizadas por la presencia de bosques de *Nothofagus*. Las tasas de recambio de OTUs microbianos y los cambios en la estructura funcional de las comunidades son menores al llegar a las etapas de sucesión avanzadas. A la vista de nuestros datos, parece que tras la aparición del bosque de *Nothofagus* existe una ralentización del recambio de especies microbianas, pero no una detención del mismo, ajustándose por ello a los principios establecidos por la Teoría Ecológica de la Sucesión y el ensamblaje de comunidades (*v.* Young et al. 2001). Sin embargo, para poder confirmar la falta de esta etapa climácica sería necesario analizar secuencias temporales más largas.

E.3 Las relaciones bióticas en la sucesión. El papel de la simbiosis

La presencia de virus a lo largo de la cronosecuencia, con familias específicamente asociadas a ciertas etapas sucesionales, sugiere que los virus están participando del control de las poblaciones de microrganismos a los que infectan, de manera semejante a lo descrito en otros ambientes (Breitbart et al. 2005; Koskella et al. 2014; Wei et al. 2015b). Además, la detección de genes de resistencia a antibióticos apunta a estos como otro posible control biótico. Dado que el papel de los antibióticos ha sido considerado más relevante en zonas de baja disponibilidad de recursos, en relación a mecanismos de competencia por ellos (Shank y Kolter 2009), estas interacciones podrían ser más importantes en las etapas iniciales de la sucesión.

El comienzo de la colonización del suelo por parte de organismos macroscópicos, líquenes y briófitos, e inmediatamente después por *Gunnera magellanica*, coincide con la desaparición de cianobacterias en los suelos. Sin embargo, tanto muchas de las especies de líquenes presentes en estas etapas intermedias (*Placopsis* spp., *Stereocaulon* spp.) (de los Ríos et al. 2011; Raggio et al. 2012), como los briófitos (Arróniz-Crespo et al. 2014) y *Gunnera magellanica*, poseen cianobacterias asociadas de los mismos géneros que los detectados en los suelos de las etapas iniciales. Mediante el establecimiento de estas interacciones bióticas, las cianobacterias ocupan hábitats más estables, estando aparentemente más protegidas frente a parásitos y patógenos (*e.g.* hongos *Chytridiomycetes*, virus) o frente a organismos ramoneadores (*e.g.* protozoos, microinvertebrados). Además, parece claro que siguen jugando un papel fundamental para el ecosistema gracias a su capacidad de fijar N_2 atmosférico e incorporarlo al sistema (DeLuca et al. 2008; Osborne y Bergman 2009; Zackrisson et al. 2009; Arróniz-Crespo et al. 2014). Algunos de los líquenes presentes en las etapas intermedias de la sucesión, especialmente *Placopsis pycnotheca*, especie primocolonizadora muy abundante en la zona, y otras especies con cianobacterias asociadas como *Stereocaulon* spp., pueden jugar un papel clave en los procesos sucesionales, ya que su presencia aumenta la estabilidad y la capacidad de retención de agua de los suelos, al tiempo que facilitan la incorporación al medio de carbono y de nitrógeno (de los Ríos et al. 2011; Raggio et al. 2012). Por otro lado, las cianobacterias asociadas con briófitos presentan unas altas tasas de fijación de nitrógeno (Arróniz-Crespo et al. 2014), las cuales contribuyen también al desarrollo y colonización del suelo. Por último, la relación simbiótica *Gunnera-Nostoc* favorece también la incorporación de estos nutrientes esenciales al sistema. Por todo ello, las relaciones simbióticas parecen tener una gran relevancia en la sucesión, contribuyendo al desarrollo del suelo, y, por tanto, facilitando el establecimiento de otros organismos macroscópicos en fases posteriores, lo cual ha sido puesto de manifiesto en numerosos estudios en áreas similares (Chapin 1994; Schwencke y Carú 2001; Guevara et al. 2002; Densmore 2005; Schulz et al. 2013).

El desarrollo de las comunidades vegetales en las áreas deglaciadas analizadas condiciona a su vez las trayectorias y las dinámicas de colonización microbiana a lo largo de la sucesión (Chapin et al. 1994; Knelman et al. 2012; Brown y Jumpponen 2014). En este estudio se ha detectado un aumento considerable de la presencia de hongos

micorrizógenos (clase *Leotiomycetes*) asociado a la aparición de plantas de la familia *Ericaceae* (especies *Empetrum rubrum* y *Gaultheria mucronata*), con las que aparentemente forman simbiosis. Igualmente, en las etapas avanzadas de la sucesión, se detectaron una gran abundancia de la clase *Agaricomycetes*, los cuales también pueden presentar capacidades micorrizógenas. Estas asociaciones favorecen el desarrollo de las plantas implicadas, y con ello, un desarrollo del suelo que también facilita la colonización posterior de otros organismos (Jumpponen 2003; Brown y Jumpponen 2014; van der Putten et al. 2013; Thébault et al. 2014; Powell y Klironomos 2014).

Las interacciones bióticas podrían contribuir a la ralentización observada en los procesos de recambio de especies una vez que *Nothofagus* empieza a establecerse en la cronosecuencia (*v.* Arróniz-Crespo et al. 2014; Thébault et al. 2014). Plantas y microorganismos compiten por los mismos recursos en estas fases avanzadas de la sucesión. Así, arbustos y *Nothofagus* podrían inhibir la presencia de ciertos microorganismos edáficos al captar la gran mayoría de los nutrientes presentes o al reducir la cantidad de luz que llega a la superficie del suelo. Estas mismas plantas pueden, a su vez, producir compuestos vegetales alelopáticos (Tollivier et al. 1995). Sin embargo, no es posible determinar si estos mecanismos de inhibición promueven realmente un recambio de especies o simplemente ralentizan las dinámicas sucesionales, de manera semejante a lo propuesto en otras áreas glaciares estudiadas (Walker y Chapin 1986; Farrel 1991; Matthews 1992; Chapin 1994).

La sucesión primaria que tiene lugar en las áreas descubiertas tras el retroceso de los glaciares estudiados en Tierra del Fuego se caracteriza por un rápido recambio de taxones de microorganismos y un perfil funcional diferenciado determinado por estos cambios. Las variaciones sucesionales detectadas podrían estar encaminados al máximo aprovechamiento de los recursos disponibles en cada etapa, mientras que las dinámicas que presentan se ven muy influenciadas por factores abióticos como las precipitaciones. Por otro lado, las interacciones bióticas podrían tener un papel clave en la sucesión, en especial el establecimiento de asociaciones simbióticas. La simbiosis favorece la colonización del suelo por parte de los organismos implicados, facilitando también la

colonización posterior de otros organismos, gracias a su papel en la movilización de nutrientes y a los cambios estructurales que producen en el suelo.

CONCLUSIONES

"Preferiría tener preguntas que no puedan ser respondidas

antes que respuestas que no puedan ser cuestionadas"

Richard Feynman

F. CONCLUSIONES

1- La sucesión en los terrenos recientemente deglaciados estudiados en esta tesis doctoral comienza con unas comunidades de microorganismos muy distintos a lo encontrados en fases posteriores, lo que parece indicar el establecimiento de ciertos organismos capaces de vivir exclusivamente en estos ambientes oligotróficos.

2- La sucesión primaria, en los dos glaciares de Tierra del Fuego estudiados, comienza con etapas iniciales que presentan una composición taxonómica de bacterias, hongos y algas muy similar, caracterizada por la presencia de grupos como la clase de bacterias *Cyanobacteria*, la de hongos *Chytridiomycetes* o el orden de algas *Prasiolales*. Sin embargo, posteriormente, las diferencias entre ambos glaciares en términos de diversidad se hacen más notables, si bien las tendencias generales en los grandes grupos se mantiene, con un incremento de la abundancia de las clases bacterianas *Alphaproteobacteria* y *Acidobacteria*, de clases de hongos micorrícicos y de órdenes de algas como *Microthamniales* y *Chlamydomonadales*.

3- Las diferencias en los procesos de sucesión entre ambos glaciares son particularmente notables respecto a la dinámica temporal, siendo las tasas de recambio de especies (unidades taxonómicas) mucho más elevadas en el glaciar estudiado de la vertiente sur de la Cordillera Darwin, de forma similar a los datos disponibles para la sucesión vegetal, y reflejando la posible influencia de los factores abióticos (mayores precipitaciones).

4- Las bacterias y los hongos podrían tener un papel más relevante en los procesos de sucesión que las algas, ya que la distribución de los distintos grupos taxonómicos bacterianos y fúngicos está específicamente ligada al estado de desarrollo del suelo, mientras que los grupos de algas fueron predominantemente ubicuos. Mientras que las bacterias parecen tener mayor

influencia en las etapas iniciales, en las cuales participan en rutas metabólicas de incorporación de nutrientes al medio, los hongos podrían alcanzar mayor relevancia en las etapas sucesionales intermedias y avanzadas, por su capacidad de degradar materia orgánica y formar asociaciones simbióticas con plantas vasculares y algas.

5- La estructura funcional de las comunidades microbianas del Glaciar Pía cambia en paralelo a la sucesión. Los microorganismos de etapas sucesionales iniciales podrían estar participando de rutas que incrementan la disponibilidad de nutrientes en el suelo, mientras transformaciones con requerimientos más específicos, como la desnitrificación y la metanogénesis, y, posteriormente, la degradación de sustratos orgánicos complejos, parecen tener un papel más relevante en etapas sucesionales intermedias y avanzadas, con abundante colonización vegetal.

6- Las actividades metabólicas de los microorganismos que colonizan los terrenos estudiados juegan un papel clave en el reciclado de nutrientes a lo largo de la sucesión, ya que genes de rutas metabólicas secuenciales dentro del ciclo del nitrógeno y del carbono se asocian a estados de sucesión consecutivos, existiendo una correlación con cambios en atributos del suelo. Por tanto, la disponibilidad de nutrientes parece el motor clave en la funcionalidad microbiana de estos suelos.

7- La presencia de genes de resistencia a antibióticos y las variaciones en la diversidad de virus, junto con los mecanismos de facilitación (simbiosis), a lo largo de la cronosecuencia reflejan la influencia de los factores bióticos en la sucesión.

8- La sucesión microbiana muestra una ralentización de las tasas de recambio taxonómico y una disminución de la diferenciación funcional que coincide con el inicio de la colonización por *Nothofagus* spp. y el desarrollo del bosque maduro.

9- *Gunnera magellanica* se asocia a un amplio rango de filotipos de *Nostoc* en el área de estudio, lo cual parece conferirle la capacidad de establecerse en suelos oligotróficos. El estudio de la variabilidad genética de los microsimbiontes a varias escalas reveló que las colonias presentes dentro de la misma planta no difieren entre sí, pero sí entre localidades, especialmente entre las correspondientes a distintos estadios de sucesión y, sobre todo, las procedentes de las dos vertientes de la Cordillera Darwin, sujetas a distintos régimen de precipitaciones.

10- La simbiosis parece jugar un papel fundamental en los cambios en la trayectoria y dinámica de la sucesión en las áreas deglaciadas estudiadas, ya que hongos formadores de líquenes (*Lecanoromycetes*), así como microorganismos en simbiosis con plantas vasculares (*Nostoc* y hongos micorrizógenos como *Leotiomycetes* y *Agaricomycetes*), están muy extendidos en las etapas intermedias y avanzadas de la sucesión.

11- Los grupos de microorganismos estudiados presentan patrones de diversidad específicos a lo largo de las cronosecuencias estudiadas, con tasas de recambio determinadas en gran manera por factores abióticos. Esta diferente estructura de la comunidad, y los cambios funcionales observados, parecen estar influidos por la disponibilidad de nutrientes en el medio. Las interacciones bióticas podrían también participar en estos procesos de sucesión.

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“En algún sitio,

algo increíble espera ser descubierto”

Carl Sagan

Madrid, junio de 2016